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3. Claims 1, 25, and 26 Are Patentable

The Action rejects claims 1, 25, and 26 under 35 U.S.C. § 103 (a) as being unpatentable over Kubota, in view of Frisch *et al.*. Applicants respectfully traverse this rejection.

In view of the Declaration of John A. Copland under 37 C.F.R. § 1.131, Applicants contend that Kubota is not a proper prior art references. As argued above, Applicants contend that a proper *prima facie* rejection for obviousness cannot be made because all of the claim elements are not taught by Fritsch *et al.* Accordingly, Applicants request this rejection be withdrawn.

4. Claims 1, 28-31, 36, and 38 Are Patentable

The Action rejects claims 1, 28-31, 36, and 38 were all rejected under 35 U.S.C. § 103 (a) as being unpatentable over Kubota, in view of Roth *et al.*. Applicants respectfully traverse this rejection.

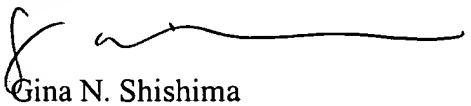
In view of the Declaration of John A. Copland under 37 C.F.R. § 1.131, Applicants contend that Kubota is not a proper prior art references. Applicants reiterate the arguments made above that the claimed invention is not taught by the remaining reference of Roth *et al.* This reference does not teach or suggest a thiazolidinedione compound, which is recited by claims. As this is not a proper *prima facie* rejection, Applicants respectfully request it be withdrawn.

F. Conclusion

Applicants believe that the present document is a full and complete response to the referenced Official Action. In conclusion, Applicants submit that, in light of the foregoing remarks, the present case is in condition for allowance and such favorable Action is respectfully

requested. Should the Examiner have any further questions or comments, or believe that certain clarifications might more readily progress the present application to issuance, a telephone call to the undersigned Applicants' representative at (512) 536-3081 is earnestly solicited.

Respectfully submitted,



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Date: January 15, 2001

APPENDIX A:
Claims Pending as of Office Action Dated 8/16/00

1. (Amended) A method for inhibiting the growth of a cancer cell comprising

(i) contacting the cancer cell with a thiazolidinedione compound; and

(ii) contacting the cancer cell with a chemotherapeutic drug or irradiating the cancer cell with X-ray irradiation, UV-irradiation, γ-irradiation, or microwaves,

in amounts effective to inhibit the growth of the cancer cell.

2. The method of claim 1, wherein the thiazolidinedione compound is a troglitazone.

3. The method of claim 1, wherein the thiazolidinedione compound is a pioglitazone.

4. The method of claim 1, wherein the thiazolidinedione compound is a rosiglitazone.

5. The method of claim 1, wherein the cell is a mammalian cell.

6. The method of claim 5, wherein the cell is a human cell.

7. The method of claim 1, wherein the contacting occurs *in vitro.*

8. The method of claim 1, wherein the contacting occurs *in vivo.*

9. The method of claim 1, wherein the cell is selected from a group consisting of a bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestine, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, and uterus cell.

10. The method of claim 9, wherein the cell expresses PPAR-γ.

11. The method of claim 9, wherein the cancer cell is a bone cancer cell.

12. The method of claim 11, wherein the bone cancer cell is an osteosarcoma cell.

13. The method of claim 11, wherein the cell is a precursor to osteosarcoma.

14. The method of claim 9, wherein the cancer cell is an ovarian cancer cell.

15. The method of claim 9, wherein the cancer cell is a renal cancer cell.

16. (Amended) The method of claim 1, wherein the cancer cell is contacted with a chemotherapeutic drug.

17. The method of claim 16, wherein the chemotherapeutic drug comprises an alkylating agent, mitotic inhibitor, antibiotic, nitrosurea, antimetabolite, corticosteroid hormone, or other antineoplastic agent.

18. The method of claim 17, wherein the chemotherapeutic drug comprises an alklyating agent.

19. The method of claim 17, wherein the chemotherapeutic drug comprises a mitotic inhibitor.

20. The method of claim 17, wherein the chemotherapeutic drug comprises an antibiotic.

21. The method of claim 17, wherein the chemotherapeutic drug comprises a nitrosourea.

22. The method of claim 17, wherein the chemotherapeutic drug comprises an antimetabolite.

23. The method of claim 17, wherein the chemotherapeutic drug comprises a corticosteroid hormone.

24. The method of claim 17, wherein the chemotherapeutic drug comprises an antineoplastic agent.

25. The method of claim 1, wherein the thiazolidinedione compound is contacted with a cancer cell by administering the thiazolidinedione regionally, endoscopically, intravenously, intralesionally, percutaneously, subcutaneously, intraperitoneally, intratracheally, intramuscularly, or by perfusion.

26. The method of claim 17, wherein the thiazolidinedione and the chemotherapeutic drug are suitably dispersed in a pharmacologically acceptable formulation.

27. The method of claim 1, wherein the thiazolidinedione compound is contacted with the cell at the same time as contact with the chemotherapeutic agent.

28. The method of claim 1, wherein the cancer cell is a tumor cell in a tumor.

29. The method of claim 28, further comprising resecting the tumor.

30. (Amended) The method of claim 28, wherein the cancer cell is irradiated with X-ray irradiation, UV-irradiation, γ-irradiation, or microwaves.

31. The method of claim 30, wherein the thiazolidinedione compound is contacted with the cell at the same time as irradiation.

32. (Amended) The method of claim 25, further comprising contacting the cell with a therapeutic polynucleotide selected from the group consisting of a Dp gene, p21, p16, p27, E2F, Rb, APC, DC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, *ras*,

myc, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl*, *abl*, Bax, Bcl-X_s and E1A; wherein the therapeutic polynucleotide is expressed in the cell.

33. A method for treating cancer in a patient comprising administering to the patient troglitazone and a chemotherapeutic drug in an amount effective to inhibit the cancer.

34. A method for inhibiting the cell cycle progression of a mammalian cancer cell comprising contacting the cell with an amount of troglitazone effective to inhibit the cell cycle progression of the cell.

35. A method of treating cancer in a patient comprising administering to the patient a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

36. (Amended) A method for treating microscopic residual cancer comprising the steps of:

(i) identifying a patient having a resectable tumor;

(ii) resecting said tumor; and

(iii) contacting the tumor bed with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

37. (Amended) A method for treating a subject having a tumor comprising the steps of:

(i) surgically revealing said tumor; and

(ii) contacting said tumor with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

38. (Amended) A method for treating a subject having a tumor comprising the step of perfusing said tumor, over an extended period of time, with a therapeutically effective amount of troglitazone and a chemotherapeutic drug.

39. The method of claim 27, wherein the thiazolidinedione and the chemotherapeutic agent are combined in a therapeutic formulation.

40. A method for inhibiting the growth of a cancer cell comprising i) contacting the cancer cell with a composition comprising troglitazone and ii) contacting the cancer cell with a chemotherapeutic agent or irradiating the cancer cell, in amounts effective to inhibit growth of the cancer cell.

41. The method of claim 40, wherein the cancer cell is contacted with a chemotherapeutic agent.

42. The method of claim 41, wherein the composition comprises troglitazone and a chemotherapeutic agent.

43. The method of claim 40, wherein the cancer cell is a bone cancer cell.

44. The method of claim 43, wherein the bone cancer cell is an osteosarcoma cell.

45. The method of claim 40, wherein the cancer cell is an ovarian cancer cell.

46. The method of claim 40, wherein the cancer cell is a renal cancer cell.

CURRICULUM VITAE

NAME: John Alton Copland, III DATE: October 5, 2000

PRESENT POSITION AND ADDRESS:
Research Assistant Professor
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EDUCATION:

1975-1979	B.S. Chemistry, Columbus College, Columbus GA.
1979-1983	M.S., Department of Endocrinology, MCG, Augusta GA.
1987-1992	Ph.D., Department of Physiology and Endocrinology, Medical College of Georgia (MCG), Augusta GA.
1992-1995	Postdoctoral Fellow, Department of Internal Medicine, Endocrine Section, University of Texas Medical Branch, Galveston TX.
1995-1999	Postdoctoral Fellow, Department of Obstetrics & Gynecology, University of Texas Medical Branch, Galveston TX.
1999-present	Research Assistant Professor, Department of Internal Medicine, Endocrine Section, University of Texas Medical Branch, Galveston TX.

RESEARCH ACTIVITIES:
A. Area of Research
-Interaction of transcriptional factors with their cognate response elements.
-Analysis of the gene regulation and function of oxytocin receptor gene
-Functional analysis of the steroid receptors.
-Cell signalling responses activated through cell surface membrane receptors.
-Mechanism of action of growth inhibitory factors on tumor cell proliferation and differentiation

B. Grant Support
-William and Mary Research Fund (1996-1997)
-John Sealy Memorial Recruitment Grant "Thiazolidinediones (TZDs): Activators of peroxisome proliferator-activated receptory (PPARγ) and mediators of anti-tumoral activity in renal cell carcinoma (11/2000 – 11/2002)
-American Cancer Society Institutional Research Grant "Cross talk between PPAR gamma and PKCs" (11/2000 – 10/2001)

COMMITTEE RESPONSIBILITIES:
A. National:
Inaugural Chairperson, American Association for Cancer Research (AACR) Associate Member Council (1996-1998).

Member, AACR Associate Member Council (1996-1999).
B. UTMB:
C. Departmental:
Member of strategic planning committee for Division of Reproductive Science, Dept. Ob/Gyn (1996)

TEACHING RESPONSIBILITIES AT UTMB:

1. Journal Club presentations in Braiser Laboratory of the Endocrine Section of Internal Medicine (1992-1995).
2. Journal Club presentations in Sealy Center for Molecular Sciences (1992-1995).
3. Graduate student preceptor for graduate students rotating through Dr. Brasier's laboratory (1993-1995).
4. Sealy Center for Molecular Science, "In vivo footprinting to study the interaction of inducible transcription factors", May 24, 1993.
5. Division of Reproductive Science Workshop, "Promoter expression studies on the human oxytocin receptor gene", February 7,1996.
6. Division of Reproductive Science Research Forum, "Regulation of the oxytocin receptor in the Hs578T human mammary tumor cell line", September 3, 1996.
7. Division of Reproductive Science Research Forum,"Oxytocin receptor regulation in a mammary cell tumor cell line, MCF-7", September 2, 1997.
8. Division of Reproductive Science Research Forum,"AP1 and ETS transcriptional factors synergistically upregulate OTR in Hs578T mammary tumor cells", September 1, 1998.
9. Ob/Gyn Fellow preceptor in Dr. Soloff's laboratory 1997-1999.
   Division of Endocrinology, Internal Medicine Seminar Series, "Thiazolidinediones in the treatment of cancer". August 4, 1999.
10. Endocrine Research Seminar, "Thiazolidinediones in the treatment of cancer" (August 4, 1999).
11. Problem Based Learning facilitator for Endocrine Teaching Block for 2nd year medical students (1999-2000).
12. Mentor medical student (Patti DeGroot) in work study program (2000).
13. Coordinator for Bimonthly Research Seminar Series sponsored by Endocrinology Division, UTMB
13. Small groups discussion leader for first year graduate students of two Science journal articles-October 20, 2000.

MEMBERSHIP IN SCIENTIFIC SOCIETIES:
Associate Member American Association for Cancer Research (1991-2/2000)
Associate Member of Endocrine Society (1997-2/2000)
Member American Association for Cancer Research (2/2000-present)
Member Endocrine Society (2/2000-present)

HONORS:
Cancer grant from the Fraternal Order of Eagles (1986-1987)
Excellence in Research Award, Medical College of Georgia (1990).
National Research Service Award (1991-1992).
Grant reviewer for Susan G. Komen Breast Cancer Foundation Postdoctoral Fellowships (May, 1998)

Grant reviewer for Susan G. Komen Breast Cancer Foundation for Senior Investigator grant awards (August, 1998)
Reviewer for *Biology for Reproduction* (1999-present)
Ad hoc reviewer for *International Journal of Cancer* (2000)
Grant reviewer for Susan G. Komen Breast Cancer Foundation (February, 2000)

ADDITIONAL INFORMATION:
Skills:
-Recombinant bacterial expression and purification of proteins *i.e.*, glucocorticoid receptor and heat shock proteins.
-Studied interaction of the glucocorticoid receptor with the angiotensinogen promoter.
-Utilized cell culture techniques and *in vivo* animal models to examine growth inhibitory effects of antitumor agents.
-Techniques include RIA, steroid receptor assays, *in vivo* and *in vitro* footprinting, stable and transient transfections, gel mobility shift assays, westerns, northerns, southerns, ribonuclease protection assays, RT-PCR, DNA sequencing, and plasmid vector construction.

Accomplishments:
1. Masters Thesis: Ovarian factors affecting the postovulatory follicle stimulating hormone surge in the New Zealand whit rabbit (1983).
2. Ph.D Dissertation: Antitumor activity of 3-phenylacetylamino-2,6-piperidinedione and its computer modeled analogs (1992).
3. AACR Associate Member Council Activities (1997-8): Organized programs for the associate members of AACR (>4200 members) for the AACR annual meeting i.e. grant writing workshop and networking sunrise session. The Council raised $285, 000 to support these programs as well as Associate Member newsletter, travel awards (>100 at $1000/awardee) to young scientists in training to attend annual AACR meeting and special AACR conferences, and a two year grant for young scientist moving into first faculty appointment. Also, crafted a recruitment brochure and started a newsletter for AACR Associate Members. Moderator of Sunrise Session at AACR Annual Meeting (1997) entitled, Making Sound Career Choices: Advice from the Experts. Organized public forum on Cancer Diagnosis, Treatment, and New Clinical Trials presented in Columbus, Georgia on September 18, 1998 by members of the Associate Member Council. Also, started the AACR Associate Member Newsletter (1998) with the latest contributed article to the Associate Member News (Spring 2000) entitled, *The AACR Annual Meeting Challenge: Preparation + Practice = Success.*
4. Patent pending No. PCT/US98/20611. September 29, 1999
5. Patent application filed October 29,1999 , Serial No. 09/430,114.
6. Patent application filed October 29,1999 , Serial No. 09/418,095.

PUBLISHED:
A. ARTICLES IN PEER-REVIEWED JOURNALS:
1. Mills, T., Copland, A. and Osteen, K. Factors affecting the postovulatory surge of FSH in the rabbit. Biol. Reprod. 25:530-535, 1981.
2. Mills, T.M. and Copland, J.A. Effects of ketamine-xylazine anesthesia on blood levels of luteinizing hormone and follicle-stimulating hormone in the rabbit.

Lab. Anim. Sci. 32:619-621, 1982.
3. Mills, T.M. and Copland, J.A. Effects of castration and estradiol treatment on the postovulatory secretion of follicle-stimulating hormone in the mated rabbit. Biol. Reprod. 28:306-311, 1983.
4. Mills, T.M., Copland, J.A., Coy, D.H. and Schally, A.V. Is the postovulatory secretion of follicle-stimulating hormone mediated by LHRH? Endocrinology 113:1020-1024, 1983.
5. Smanik, E.J., Copland, J.A. and Muldoon, T.G. Cryptic estrogen binding protein complicates analysis of estrogen receptor distribution. Biochem. Biophys. Res. Commun. 125:557-583, 1984.
6. Copland, J.A., Smanik, E.J. and Muldoon, T.G. Discrete early changes in cellular subpopulations of rat uterine and anterior pituitary estrogen receptor in response to acute exposure to exogenous estradiol. J. Steroid Biochem. 26:723-731, 1987.
7. Wood, J.C., Copland, J.A., Muldoon, T.G. and Hendry, L.B. 3-Phenylacetylamino -2,6-piperidinedione inhibition of rat Nb2 lymphoma cell mitogenesis. Proc. Soc. Expl. Biol. Med. 197:404-408, 1991.
8. Copland, J.A., Hendry, L.B., Chu, C.K., Wood, J.C., Pantazis, C.G., Wrenn, R.W. and Mahesh, V.B. Inhibition of estrogen stimulated mitogenesis by 3-phenylacetyl-amino-2,6-piperidinedione and its p-hydroxy analog. J. Steroid Biochem. and Molec. Biol. 46:451-462, 1993.
9. Hendry, L.B., Chu, C.K., Copland, J.A. and Mahesh, V.B. Antiestrogenic piperidinediones designed prospectively using computer graphics and energy calculations of DNA/ligand complexes. J. Steroid Biochem. and Molec. Biol. 48:495-505, 1994.
10. Hendry, L.B., Chu, C.K., Rosser, M.L., Copland, J.A., Wood, J.C. and Mahesh, V.B. Design of novel antiestrogens. J. Steroid Biochem. and Molec. Biol. 49:269-280, 1994.
11. Jeng, Y.-J., Lolait, S.J., Strakova, Z., Chen, C., Copland, J.A., Mellman, D., Hellmich, M.R., and Soloff, M.S. Molecular cloning and functional characterization of the oxytocin receptor from a rat pancreatic cell line (RINm5F). Neuropeptides 30:557-565, 1996.
12. Strakova Z., Copland J.A., Lolait S.J., and Soloff M.S. ERK2 mediates oxytocin-stimulated PGE2 synthesis. Am J Physiol 274:E634-E641, 1998.
13. Hoare, S., Copland, J.A., Wood, T.G., Jeng, Y-J., Izban, M.G., Soloff, M.S. Characterization of human oxytocin receptor gene promoter. Endocrinology, 140:2268-2279, 1999.
14. Copland, J.A., Ives, K., Jeng, J-Y., Strakova, Z., Hellmich, M.R., Soloff, M.S. Protein kinase C: A key link in gene regulation and function of oxytocin receptor in the human mammary Hs578T cells. Endocrinology 140:2258-2267, 1999.
15. Copland, J.A., Simmons, D., Ives, K., and Soloff, M.S. Discovery of functional oxytocin receptors in human osteoblasts. Endocrinology 140:4371-4374, 1999.
16. Hoare, S., Copland, J.A., Strakova, Z., Ives, K., Jeng, J-Y, Hellmich, M., Soloff, M. Proximal Portion of the COOH Terminus of the Oxytocin Receptor Is Required for Coupling to G_q, but G_i. INDEPENDENT MECHANISMS FOR ELEVATING INTRACELLULAR CALCIUM CONCENTRATIONS FROM INTRACELLULAR STORES . J. Biol. Chem. 274: 28682-28689, 1999.

17. Zlatnik, M., Copland, J.A., Ives, K., and Soloff, M.S. Functional oxytocin receptors in a human endometrial cell line. Am J of Obstet and Gynecol 182:850-855, 2000
18. Ma, T, Copland, JA, Brasier, AR, Thompson, EA. A novel glucocorticoid receptor binding element within the murine c-myc promoter. Mol Endo 14:1377-1386. 2000.

B. OTHER

Invited Chapters:

1. Muldoon, T.G., Watson, G.H., Smanik, E.J. and Copland, J.A. Regulation of steroid hormone receptor nature and concentration as a function of normal and abnormal hormonal environments. In: Hormonally Responsive Tumors. Editor, Vincent P. Hollander, Academic Press, Inc., New York, pp. 237-258, 1985.
2. Brasier, A.R., Li, J., and Copland, A. Transcription factors modulating angiotensinogen gene expression in hepatocytes. Kidney International. 46:1564-1566, 1994.
3. Soloff, M.S., Jeng, J-Y., Copland, J.A., Hoare, S., Strakova, Z. Signal pathways mediating oxytocin stimulation of prostaglandin synthesis in selected target cells. Experimental Physiology 85S:51S-58S, 2000.
4. Cesen-Cummings K., Copland, J.A., Barrett, J.C., Walker, C.L., and Davis, B.J. Pregnancy, parturition, and prostaglandins: Defining uterine leiomyomas. 108 Suppl 5:817-820, 2000.

Invited Lectures:

1. Rutgers University, Department of Animal Sciences, New Brunswick, New Jersey, "The effects of a novel antitumor agent upon estrogen receptor levels in breast cancer", October 23, 1992.
2. 18th International Congress of Chemotherapy, Stockholm, Sweden, "Antiestrogenic effects of 3-phenylacetylamino-2,6-piperidinedione and its para-hydroxy analog in MCF-7 (E3) breast tumor cells", July 1, 1993.
3. Medical University of South Carolina, Department of Cell Biology and Anatomy, Charleston, South Carolina, "Functional glucocorticoid receptor overexpressed in E. Coli", February 23, 1995.
4. Public Education Forum on Cancer Diagnosis, Treatment, and New Clinical Trials. Hilton Hotel, Columbus, Georgia "Ovarian Cancer Diagnosis, Treatment, and New Clinical Trials" on September 18, 1998. Organized by members of the AACR Associate Member Council and AFLAC, Incorporation.
5. Bassett Healthcare Research Institute, Cooperstown, New York, "Gene regulation and function of the oxytocin receptor in human mammary tumor cells". October 23, 1998.
6. University of Texas Medical Branch, The Marine Biomedical Institute and Department of Anatomy & Neurosciences, Galveston Texas, "Thiazolidinediones in the treatment of cancer". October 18, 2000.

C. ABSTRACTS:

1. Mills, T., Osteen, K. and Copland A. Factors controlling the postovulatory secretion of FSH in the rabbit. Endocrinology 108(S)P:335, 1980.
2. Copland, J.A., Mills, T.M., and Muldoon, T.G. Luteinizing hormone releasing hormone and follicle stimulating hormone secretion in the postovulatory rabbit. Society of Experimental Biology and Medicine Southeastern Section. 7:10, 1982.
3. Mills, T.M. and Copland, J.A. The effects of porcine follicular fluid inhibition

on gonadotropin secretion in the rabbit. Endocrinology. 112(S):114, 1983.
4. Copland, J.A., Smanik, E.J. and Muldoon, T.G. Uterine and anterior pituitary estrogen receptors revisited: The "spurious forms". Endocrinology 114(S):8, 1985.
5. Copland, J.A., Hendry, L.B., and Muldoon, T.G. Examination of unoccupied nuclear estrogen receptor turnover as an indicator of cellular receptor localization *in vivo* and *in vitro*. Society of the Study of Reproduction. 30(S):235, 1986.
6. Copland, J.A. and Muldoon, T.G. Differential inhibitory properties of 3-phenylacetylamino-2,6-piperidinedione and its major metabolites on cell growth of tumor cell lines. Southeastern Endocrine Society. Atlanta GA, Abstract #11, 1989.
7. Copland, J.A., Wood, J.C., Hendry, L.B., Pantazis, C.G., Chu, C.K., and Mahesh, V.B. In vitro and in vivo inhibition of estrogen induced tumor growth by a novel antitumor compound. Annual Meeting of the American Association for Cancer Research. Houston TX, Abstract #783, 1991.
8. Copland, J.A., Wood, J.C., Wrenn, R.W., and Hendry, L.B. Action of an amino acid analog (3-phenylacetylamino-2,6-piperidinedione) and tamoxifen in inhibiting cell growth in vitro. Society for the Study of Reproduction. 35(S):270, 1991.
9. Copland, J.A., Wood, J.C., Chorich, L.P., Wrenn, R.W., Pantazis, C.G., Hendry, L.B., and Mahesh, V.B. Action of estradiol and 3-phenylacetylamino-2,6-piperidinedione upon mitogenesis, steroid receptor levels and protein kinase A and C activity in vitro. An American Association for Cancer Research Special Conference on Negative Controls on Cell Growth and Their Breakdown During the Pathogenesis of Cancer. Chatham MA, 1991.
10. Copland, A. and Brasier, A. Overexpression and functional characterization of the human steroid binding domain of the glucocorticoid receptor. Sealy Center for Molecular Science Forum. Galveston TX, May 15, 1995.
11. Hoare, S., Copland, A., Jeng, J., Wood, T., Izban, M., and Soloff, M. Functional analysis of the oxytocin receptor. Texas Forum on Female Reproduction. Houston TX, May 16, 1996.
12. Copland, J.A., Jeng, J-Y., Ives, K., Strakova, Z., Hellmich, M.R., Soloff, M.S. Characterization of gene regulation and function of oxytocin receptor in a human mammary myoepithelial cell line (Hs578T). Abstract Endocrine Society Meeting, Minneapolis, June, 1997.
13. Strakova, Z., Copland, J.A., Soloff, M.S. Oxytocin activates the extracellular signal regulated kinase ERK2 (p42MAPK). Abstract Texas Forum on Female Reproduction, Houston TX, May 15-16, 1997.
14. Copland, J.A., Jeng, Y-J., Ives, K., Hellmich, M.R., Soloff, M.S. Characterization of the oxytocin receptor (0TR) in human mammary Hs578T Cells. Abstract Texas Forum on Female Reproduction, Houston TX, May 15-16, 1997.
15. Copland, J.A., Jeng, Y-J., Ives, K., Hellmich, M.R., Soloff, M.S.Regulation and function of the oxytocin receptor (OTR) in human mammary Hs578T cells. Sealy Center for Molecular Science Forum. Galveston TX, May 30, 1997. Abstract #36.
16. Hoare, S., Copland, A., Acosta, M., Jeng, J., Wood, T.G., Izban, M.G., and Soloff, M. Regulatory element in the proximal promoter region activates transcription of human oxytocin receptor gene. Sealy Center for Molecular Science Forum. Galveston TX, May 30, 1997. Abstract #38.

17. Strakova, Z., Copland, J.A., Soloff, M.S. Oxytocin activates the extracellular signal regulated kinase ERK2 (p42MAPK). Sealy Center for Molecular Science Forum. Galveston TX, May 30, 1997. Abstract #39.
18. Copland, J.A., Zlatnik, M., Ives, K., Soloff, M.S. Characterization of oxytocin receptor in a human granulosa cell line. Society for Gynecological Investigation. Atlanta, GA March 11-14, 1998.
19. Hoare, S., Copland, J.A., Strakova, Z., Zlatnik, M., Soloff, M.S. The importance of the C-terminal tail of the rat oxytocin receptor in signal transduction. Society for Gynecological Investigation. Atlanta, GA March 11-14, 1998.
20. Zlatnik, M.G., Copland, J.A., Ives, K., Soloff, M.S. Ishikawa: A human endometrial cell line expressing functional oxytocin receptors. Society for Gynecological Investigation. Atlanta, GA March 11-14, 1998.
21. Copland, J.A., Gasic, S., Soloff, M.S. Inhibition of cell proliferation in Saos-2 human osteosarcoma cells by troglitazone. Annual American Association for Cancer Research Meeting, New Orleans LA March 29, 1998, Abstract #475.
22. Copland, A., Soloff, M.S. Regulation of oxytocin receptor levels by PMA and cis-retinoic acid in the human mammary tumor cell line, MCF-7. Sealy Center for Molecular Science Forum. Galveston TX, May 29, 1998.
23. Hoare S., Copland J.A., Strakova Z., Ives K.L., Jeng Y.-J., Hellmich M.G., and Soloff, M.S. Elucidation of an oxytocin-receptor mediated increase in Ca2+ from intracellular stores that is PLC-independent. Society for Gynecological Investigation. Atlanta, GA March 10 - 13, 1999.
24. Copland J.A., Jeng Y.-J., Strakova Z., Ives K.L., Hellmich M.G., and Soloff, M.S. Protein kinase C: A link to regulating oxytocin receptor levels and oxytocin signaling in human breast Hs578T cells. Society for Gynecological Investigation. Atlanta, GA March 10 - 13, 1999.
25. Young, S. and Copland, J.A. Vitamin E is a potent inhibitor of uterine meiolyoma cell growth. Society for Gynecological Investigation. Atlanta, GA March 10 - 13, 1999, Abstract # 720.
26. Copland, J.A., Gasic, S., Wood, C., Sintuu, C., Soloff, MS., and Urban, R.J. Troglitazone: Inhibitor of human Saos-2 osteosarcoma cell cycle progression. American Association for Cancer Research, Philadelphia, Abstract #827, April 11, 1999.
27. Copland, J.A., Chopra, A., Peterson, J., Soloff M.S. Rapid gene induction by oxytocin in the synthesis of prostaglanin E2. Endocrine Society Meeting, San Diego, CA, Abstract #P2-338, June 13,1999.
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D. MANUSCRIPTS AND ABSTRACTS SUBMITTED, ACCEPTED, OR IN PREPARATION:

Manuscripts:

1. Copland, J.A., Chopra, A., Peterson, J., Soloff M.S. Rapid gene induction by oxytocin in the synthesis of prostaglanin E2. (In preparation).
2. Copland, J.A., Zlatnik, M., and Soloff, M.S. Oxytocin enhances progesterone release by forskolin in the HGL-5 human granulosa cell line expressing the oxytocin receptor. (Under review Biol Reprod).
3. Copland, J.A., Gasic, S., Wood, C., Sintuu, C., Soloff, M., and Urban, R. Synergy of troglitazone with 5-fluorouracil to inhibit human osteosarcoma, Saos-2, cell proliferation. (In preparation).

Abstracts:

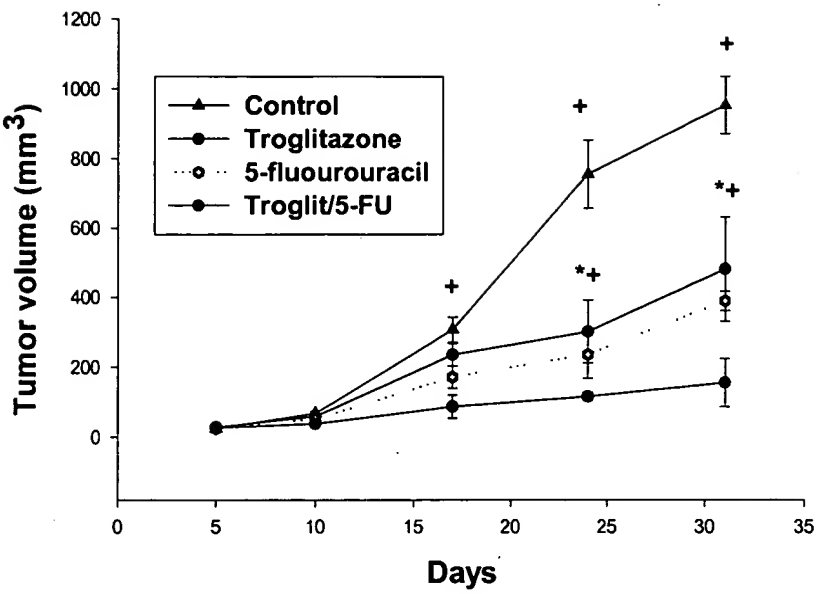
1 J.A. Copland, S. A. Gal, and J. T. Santoso. Vitamin E succinate inhibits drug-resistant human ovarian tumor growth., Society of Gynecological Oncologists, Nashville, TN, March 3-7, 2001.

2 J.A. Copland, M. Jamaluddin, T. Maity, C.G. Wood. Mechanisms by which thiazolidinediones (TZDs) mediate anti-tumoral activity in renal cell carcinoma. American Association for Cancer Research, New Orleans LA March 24-28, 2001

3 J. A. Copland, A. R. Brasier, M. Jamaluddin, E.A. Thompson, C.G. Wood. Cross talk between peroxsome proliferator-activated receptorγ (PPARγ), protein kinase C (PKC), and nuclear factor (NF)-κB. American Association for Cancer Research, New Orleans LA March 24-28, 2001

4 D.R. Fleming and J.A. Copland. Vitamin E analogs are effective inhibitors of cell proliferation in human pancreatic tumor cells. American Association for Cancer Research, New Orleans LA March 24-28, 2001

5 C. Stuart, J. Copland, T. Wood, and A. Green Human GLUT3 gene structure. Annual American Diabetes Association meeting. Philadelphia, PA June 22-26, 2001



Tumor volume of UMRC 3 tumors in nude mice.

Material & Methods:

Under sterile conditions, human renal cell carcinoma, UMRC 3 cells, were grown in culture, trypsinized, and resuspended in media at a concentration of 2 x 10^6 cell/0.1 ml. A suspension of 0.1 ml of cells were bilaterally injected subcutaneously into the trunk of 40 mice, forming two tumors per mouse. Respective treatments were begun five days after tumor implantation. Mice were either treated with troglitazone [500 mg/kg body weight (B.W.) via oral lavage Monday through Friday], 5-fluourouracil (5-FU, 10 mg/kg B.W. twice a week via lower abdominal i.p. injections); or Control (oral gavage 0.1 ml 1.5% carboxymethylcellulose/0.2% Tween 20 and i.p. injections of sterile normal saline. Each treatment were done coincidental to those of troglitazone and 5-FU). Troglitazone was suspended in sterile aqueous solution of 1.5% carboxymethylcellulose/0.2% Tween 20 and prepared fresh daily. Tumors assumed to be heli-ellipsoid in shape, were measured with calipers in three dimensions. Tumor volumes were calculated using the equation: volume (mm^3) = length x width x height x 0.5326. Statistical differences are indicated by * which is different from Control and + which is different from troglitazone/5-FU combinatorial therapy ($P<0.05$). ANOVA and Fishers PLSD were used to determine statistical differences between means of different treatment groups. Differences were considered significant at $P < 0.05$ level. Five mice per group were used. Thus, each point represents the mean +/- SD of 10 tumors.

REPORTS

Therapeutic Effect of a Retroviral Wild-Type p53 Expression Vector in an Orthotopic Lung Cancer Model

*Toshiyoshi Fujiwara, De Wei Cai, Renee N. Georges, Tapas Mukhopadhyay, Elizabeth A. Grimm, Jack A. Roth**

Background: **Mutations in the p53 tumor suppressor gene (also known as TP53) are common in human lung cancers. The wild-type form of p53 is dominant over the mutant; thus, restoration of wild-type p53 function in lung cancer cells may suppress their growth as tumors.** ***Purpose:*** **We investigated the therapeutic efficacy of direct administration of a retroviral wild-type p53 (wt-p53) expression vector (LNp53B) in an orthotopic human lung cancer model in nu/nu mice.** ***Methods:*** **Proliferation of H226Br cells was determined by cell counting after infection with LNp53B in vitro. Irradiated (350 cGy) female BALB/c nu/nu mice were inoculated intratracheally with 2×10^6 H226Br cells (whose p53 gene has a homozygous mutation at codon 254) and treated beginning 3 days later with an intratracheal instillation of LNp53B retroviral supernatant for 3 days.** ***Results:*** **Infection with LNp53B inhibited proliferation of H226Br cells in vitro. Thirty days after tumor cell inoculation, 62%-80% of the control mice showed macroscopic tumors of the right main stem bronchus. LNp53B suppressed H226Br tumor formation in 62%-100% of mice, and the effect was abrogated by dilution of the retroviral supernatant with inactive vector.** ***Conclusions:*** **Direct administration of a retroviral vector expressing wt-p53 may inhibit local growth in vivo of human lung cancer cells with abnormal p53 expression.** ***Implications:*** **Development of gene-replacement treatment strategies based on the type of mutations found in target cancers is warranted and may lead to the development of new adjunctive therapies and gene-specific prevention strategies for lung cancer. [J Natl Cancer Inst 86:1458-1462, 1994]**

Lung cancer is the single greatest cause of cancer deaths in the United States (*1*). Non–small-cell lung cancer (NSCLC), which includes squamous cell carcinoma, adenocarcinoma, and large-cell carcinoma, accounts for 75%-80% of all lung cancers (*2*). Multimodality therapeutic strategies have been applied to regionally advanced NSCLC; however, the overall cure rate, which is approximately 10%, is unsatisfactory (*3,4*).

Increased understanding of the molecular pathogenesis of cancer has profoundly changed the view of the pathogenesis of the disease; the development of cancer is now considered to result from multiple genetic alterations (*5,6*). However, the malignant phenotypes of certain cancer cells can be reversed by the introduction of a recombinant construct that reverses a single genetic lesion, a single normal cell-derived chromosome, or a copy of a wild-type tumor suppressor gene (*5,7-9*). This finding suggests that correction of a single oncogene or tumor suppressor gene abnormality may overcome the effect of multiple genetic changes in the cancer cell (*5,7-9*). Mutations of the p53 tumor suppressor gene (also known as TP53) are the genetic abnormalities most frequently identified in NSCLC (*10*). The wild-type form of p53 is dominant over the mutant. We (*11,12*) previously reported that restoration of the wild-type p53 (wt-p53) gene by a retroviral vector suppressed the growth of NSCLC cell lines in vitro and induced apoptosis in multicellular NSCLC tumor spheroids.

In the study presented here, we investigated the therapeutic efficacy of intratracheal inoculation of the wt-p53 retroviral supernatant in suppressing human NSCLC tumor formation in an orthotopic mouse model.

Materials and Methods

Cells and Culture Conditions

We routinely propagated the following two human NSCLC cell lines in monolayer culture in RPMI-1640 medium with 10% fetal calf serum (Sigma Chemical Co., St. Louis, Mo.): 1) H226Br, whose p53 gene has a homozygous mutation (mut-p53) at codon 254; and 2) H358a, a clonal derivative of H358, whose p53 gene is homozygously deleted. The H226Br cell line is a variant of the H226 cell line derived from a brain metastasis in a nu/nu mouse (gift of I. J. Fidler, The University of Texas M. D. Anderson Cancer Center, Houston). The H358 and H226 cell lines were gifts from A. Gazdar (Simmons Cancer Center, Dallas, Tex.) and J. Minna (Simmons Cancer Center) and have been previously described (*13*). The amphotropic packaging cell line GP+envAm12 and the ecotropic packaging cell line GP+E-86 (*14*) were grown in Dulbecco's modified Eagle medium (GIBCO BRL, Grand Island, N.Y.) with a high glucose concentration (4.5 g/L) supplemented with 10% newborn calf serum. For selecting GP+envAm12 cells, we used HXM medium containing hypoxanthine (15 μg/mL), xanthine (250 μg/mL), mycophenolic acid (25 μg/mL), and hygromycin B (100 μg/mL).

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See "Notes" section following "References."

Construction and Generation of a Replication-Defective Retroviral wt-p53 Expression Vector

A 1.8-kilobase (kb) wt-p53 complementary DNA (cDNA) fragment linked to a β-actin promoter was subcloned into the LNSX retroviral vector (*15*) at 3′ to 5′ orientation following removal of a simian virus 40 promoter contained in the original LNSX vector (*11*). This vector is designated LNp53B. As a control, mut-p53 cDNA (codon 273 CGT to CAT), a gift from B. Vogelstein (The Johns Hopkins University Medical School, Baltimore, Md.), was also cloned into the same retroviral vector. The GP+envAm12 cell line was transfected with the recombinant retroviral construct by the calcium phosphate coprecipitation method (*16*). The highest titer virus-producing clone was selected in medium containing the G418 antibiotic (400 μg/mL) and was then cocultured with the GP+E-86 cell line to further increase the viral titer. Following several passages over a period of 1 month, GP+envAm12 cells were selected in HXM medium, and the viral titer of the supernatant was tested by infecting NIH 3T3 cells. The titer of the retroviral supernatant was 8×10^6 colony-forming units (CFU)/mL. The supernatant was free of replication-competent virus, as assessed by an NIH 3T3 amplification assay capable of detecting five infectious viral particles per milliliter (data not shown).

Cellular RNA Extraction, Polymerase Chain Reaction, and Southern Blot Analysis to Determine Messenger RNA Expression

Total cellular RNA was isolated from monolayer cultures of virus-infected H226Br cells according to a previously described method (*17*). Briefly, cells were mixed with an equal volume of GTC solution (8 *M* guanidinium thiocyanate, 1% sarcosyl, and 0.05 *M* sodium citrate). The mixture was extracted with acid phenol–chloroform–isoamyl alcohol, and the aqueous phase was collected. RNA was precipitated with one volume of isopropanol. RNA was resuspended in 0.3 *M* sodium acetate and precipitated with two volumes of ethanol. Finally, the RNA pellet was resuspended in water treated with diethylpyrocarbonate. For cDNA synthesis, RNA samples were treated with 5 U ribonuclease-free deoxyribonuclease (Boehringer Mannheim Corp., Indianapolis, Ind.) for 1 hour at 37 °C in 0.1 *M* sodium acetate and 5 m*M* $MgSO_4$. The reaction was stopped by the addition of 10 m*M* EDTA and 0.2% sodium dodecyl sulfate. Samples were phenol extracted, precipitated with ethanol, and used as a template for cDNA synthesis from messenger RNA present in the samples.

The cDNA synthesis was done in a 20-μL reaction mixture containing 40 U RNasin (Promega Corp., Madison, Wis.), 50 m*M* Tris–HCl (pH 8.3), 75 m*M* KCl, 10 m*M* dithiothreitol, 3 m*M* $MgCl_2$, 0.1 μg oligo$(dT)_{12\text{-}18}$, 0.1 mg/mL bovine serum albumin (BSA), 0.5 m*M* of each deoxynucleoside 5′-triphosphate (dNTP), and 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL). The reaction mixture was incubated at 37 °C for 1 hour, and the enzyme was inactivated at 97 °C for 5 minutes and quickly chilled on ice. The polymerase chain reaction (PCR) was performed in a 50-μL reaction volume containing 20 μL of the reverse-transcribed samples, 0.1 mg/mL BSA, 50 m*M* KCl, 10 m*M* Tris–HCl (pH 9.0), 1.5 m*M* $MgCl_2$, 0.01% gelatin (wt/vol), 0.1% Triton X-100, 0.2 m*M* of each dNTP, 50 pmol of each of the 5′ and 3′ primers, and 1 U of Taq DNA polymerase (Promega Corp.). The amplification reaction involved denaturation at 94 °C for 1 minute, annealing at 58 °C for 1 minute, and extension at 72 °C for 1 minute using a Perkin-Elmer thermal cycler (Perkin-Elmer Corp., Mountain View, Calif.) for 35 cycles. An additional cycle for 10 minutes at 72 °C was used for complete extension. PCR products were resolved in 2% agarose TBE (1 × Tris–borate–EDTA buffer) gel and transferred onto a GeneScreen membrane (DuPont/NEN, Boston, Mass.) for Southern blot hybridization.

Cellular DNA Extraction, PCR Amplification, and Southern Blot Analysis to Determine Presence of p53 Gene

Fresh tumors were washed three times with phosphate-buffered saline and were then frozen in liquid nitrogen. High-molecular-weight DNA was extracted from frozen H226Br tumors by using Gene Releaser (BioVentures, Inc., Murfreesboro, Tenn.). One microgram of DNA was used to amplify the target gene by PCR. The specific primers used for p53 were exon 7 (5′-TCT GAC TGT ACC ACC ATC CA-3′ and 5′-CTG GAG TCT TCC AGT GTG AT-3′) and the promoter/p53 region of LNp53B (β-actin promoter: 5′-ACC TGC AGC CCA AGC TTC GAG-3′; p53 exon 4: 5′-TGC AAG TCA CAG ACT TGG CTG-3′). The PCR products were resolved on a 4% agarose gel and visualized by ethidium bromide fluorescence. For Southern blotting, the PCR products were transferred onto a GeneScreen membrane and hybridized with a ^{32}P-labeled, nick-translated p53 cDNA *Bam*HI fragment probe.

Western Blot Analysis

Total cellular protein was extracted from H358a cells infected with the LNp53B. The protein was fractionated in 10% polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was incubated with a primary antibody against p53, PAb 1801 (Oncogene Science, Manhasset, N.Y.), and then with the horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G. The ECL Chemiluminescent Western System (Amersham, Arlington Heights, Ill.) was used to detect secondary probes. Anti-actin antibody was used as a control for protein loading.

Experimental Therapy Protocol

Eighty-four 4- to 6-week-old female BALB/c nu/nu mice weighing approximately 20 g each were used in this study. The mice were provided food (Tex Lab Sterilizable Lab Blocks; Alief Feed Co., Houston, Tex.) and water ad libitum. The mice were maintained under pathogen-free conditions. All experiments involving mice were performed under protocols and guidelines approved by the M. D. Anderson Animal Care and Use Committee.

Irradiated (350 cGy) nu/nu mice were anesthetized with methoxyflurane and inoculated intratracheally with 2×10^6 H226Br cells in 0.1 mL Hanks' balanced salt solution into the right main stem bronchus via a tracheotomy. The cervical trachea was exposed by dissection of the peritracheal muscle sheath. A tracheotomy was made by puncture with a 25-gauge needle. Gentle traction on the proximal trachea with a forceps straightened the right bronchus and allowed introduction of a 27-gauge needle, which was advanced to the level of the right upper lobe orifice. The cell suspension was injected with a 1-mL syringe. Beginning 3 days after tumor cell inoculation, 0.1 mL of retroviral supernatant, mixed with 5 μg/mL protamine to enhance gene transduction (*18*), was injected once daily for 3 days through the previous tracheotomy incision using a 1-mL syringe with a 27-gauge needle. The mediastinal blocks were harvested 30 days later and assessed for tumor formation and growth by the same observer, who had no knowledge of the treatment groups. The tumor volume was calculated by measuring the perpendicular diameters with linear calipers as described previously (*19*).

Statistical Analysis

Differences in the tumor volumes for the treatment groups were compared using the Wilcoxon test. Differences in the distribution of tumor incidence among experimental groups were determined using Fisher's exact test.

Results

Expression of wt-p53 Messenger RNA in H226Br Cells and p53 Protein Production in H358a Cells

H226Br cells were transduced in vitro with the LNp53B retroviral vector by incubating 10^4 to 10^6 cells with 0.5 mL of retroviral stock in the presence of 8 μg/mL polybrene. This transinfection was repeated once daily for 3 days. We next examined whether the transduced p53 gene was expressed in these cells; the reverse transcription–PCR analysis used sense primers for β-actin promoter sequences 5′ to the promoter/p53 junctional sequences and an opposing p53 cDNA anti-sense primer located within p53 exon 4. These primers are specific for the retrovirally transduced p53. PCR products were evaluated by Southern blot hybridization with a ^{32}P-labeled, nick-translated p53 cDNA probe. A β-actin/p53 segment was detected in H226Br cells transduced with wt-p53, whereas it was not present in parental and LNSX virus-infected cells (Fig. 1). Western blot analysis demonstrated detectable levels of p53 protein following LNp53B retroviral infection in p53-negative H358a cells (data not shown).

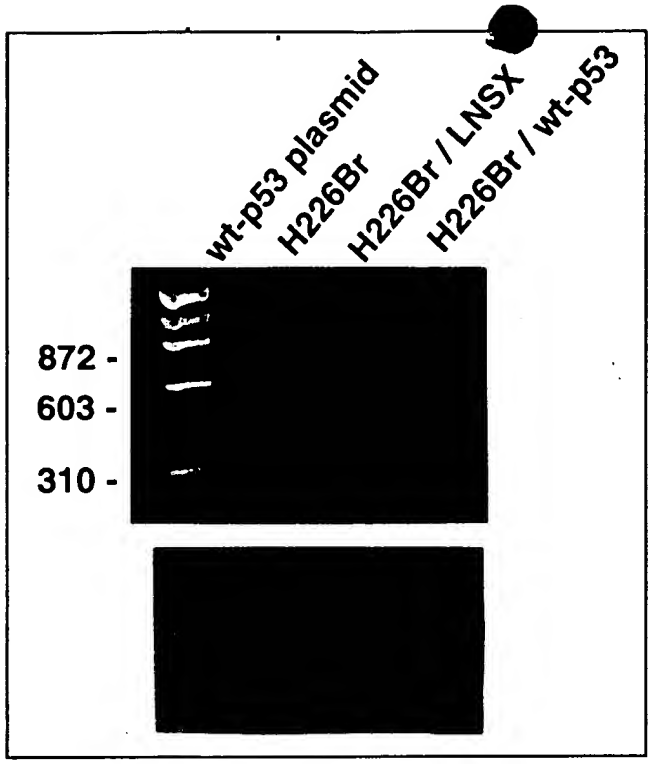

Fig. 1. Detection of transduced p53 gene expression in H226Br cells. H226Br cells were infected with the LNp53B retrovirus once a day for 3 days. First-strand cDNA generated from RNA was amplified by using β-actin promoter/p53 exon 4 primers. The PCR products were hybridized with a ^{32}P-labeled p53 cDNA probe. Upper panel, ethidium bromide gel; lower panel, Southern blot hybridization. The first lane is the molecular weight standards with sizes shown by the numbers at the left.

Inhibition of H226Br Cell Growth In Vitro After wt-p53 Transduction

Monolayer cultures of H226Br cells were exposed to LNp53B retrovirus, and the growth kinetics of the mass culture was assessed to examine the effect of in vitro transduction of wt-p53 on the growth of lung cancer cells. Infection by LNSX virus had no effect on the growth of H226Br cells, but LNp53B-infected cells showed a fourfold reduction in growth compared with parental cells (Fig. 2).

Effect of Intratracheal Instillation of wt-p53 Retrovirus in an Orthotopic Lung Cancer Model

The intrapulmonary model for the orthotopic propagation of human lung tumor cells was used to assess the effect of direct in vivo injection of the LNp53B retrovirus. H226Br tumors were successfully grown in irradiated nu/nu mice by the intratracheal inoculation of 2×10^6 tumor cells. Endobronchial tumors with local mediastinal extension were observed in approximately 75% of the animals 30 days after intratracheal inoculation (Table 1). In the therapy protocol, irradiated (350 cGy) nu/nu mice were inoculated intratracheally with H226Br cells and were then inoculated intratracheally on days 4, 5, and 6 with medium only, unmodified LNSX virus, LNp53B retrovirus, or mut-p53 retrovirus. At autopsy, 30 days after tumor inoculation, only 0%-38% of mice treated with wt-p53 retroviral supernatant had right-sided lung tumors, and these tumors were much smaller than tumors in the control groups; the effect of injecting the mut-p53 virus on in vivo tumor growth, however, was not significantly different from that in control mice (Table 1). Moreover, serial dilution of LNp53B retroviral supernatant with unmodified LNSX viral supernatant reduced the efficacy of the LNp53B virus in suppressing tumor growth (Table 1). The decreasing incidence of tumors with increasing LNp53B concentrations was statistically significant ($P<.01$ by Fisher's exact test).

The one small tumor that grew in experiment 3 (Table 1) following treatment with LNp53B retroviral supernatant was analyzed for the presence of the retroviral p53 DNA by specific PCR. PCR primers that spanned exon 4 of p53 and the β-actin promoter recognize only exogenous p53. The exogenous sequence was detected by PCR and confirmed by Southern blot hybridization with p53 cDNA in the tumor that grew following treatment with the LNp53B retroviral vector (Fig. 3). Only endogenous p53 was detected in DNA extracted from a tumor treated with the control LNSX vector.

Discussion

This study reports the efficacy of retroviral vector-mediated transfer of the wt-p53 gene into human lung cancer cells in vitro and in vivo. Expression of wt-p53 inhibited the growth of H226Br cells, which have an endogenous mut-p53. The observed growth inhibition is consistent with our previous study (*11*) in which transfection of the wt-p53 gene led to retardation of the growth of human NSCLC cell lines H322a (which have mut-p53) and H358a (which have deleted p53). These observations suggest that the introduction of wt-p53 may be an effective strategy to slow the growth and

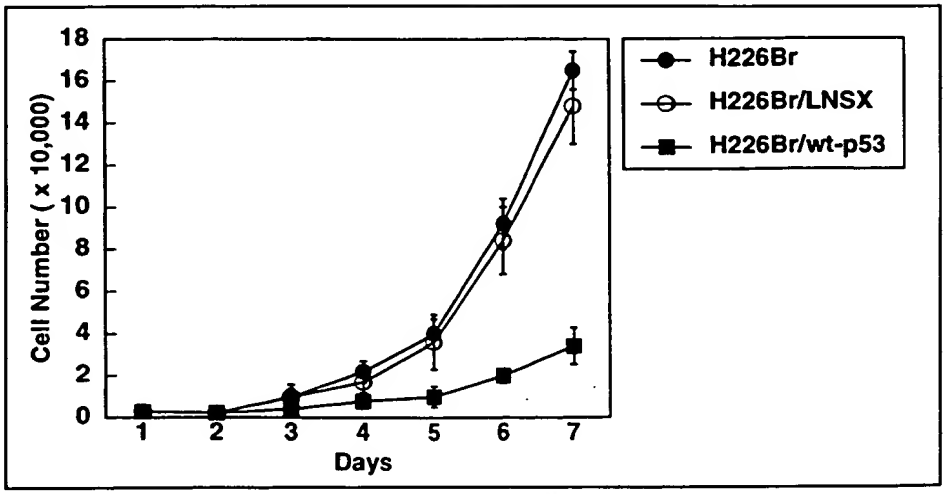

Fig. 2. Growth curve of H226Br cells transduced with the LNp53B. Equal numbers of transduced or non-transduced H226Br cells (10^4/well) were seeded in each well of a 12-well plate. Cells from three wells were trypsinized and counted by trypan blue dye exclusion at each indicated time point. The mean numbers from triplicate wells were plotted.

Table 1. Effect of wt-p53 retroviral construct on growth of H226Br tumor in nu/nu mice

Experiment No.	Treatment*	No. of mice with tumor/ total No. of mice treated	Mean volume ± SEM, mm^3†
1	Medium	7/10 (70)	2.85 ± 0.99
	LNp53B	0/8 (0)	0‡
2	Medium	7/9 (78)	2.23 ± 0.76
	LNSX only	5/7 (71)	4.47 ± 1.38
	LNp53B	3/8 (38)	0.81 ± 0.63‡
	mut-p53	5/8 (62)	3.78 ± 1.14
3	LNSX only	8/10 (80)	1.43 ± 0.81
	LNSX/LNp53B (3:1)	4/6 (67)	1.55 ± 1.01
	LNSX/LNp53B (1:1)	3/8 (38)	2.26 ± 0.89
	LNp53B	1/10 (10)§	0.33‡

*Irradiated (350 cGy) nu/nu mice were inoculated intratracheally in the right main stem bronchus with 2×10^6 H226Br cells. On days 4, 5, and 6, mice were treated with intratracheal inoculation (0.1 mL) of either medium or LNSX, LNp53B, or mut-p53 retroviral supernatants. In experiment 3, mixed solutions of LNSX and LNp53B viral supernatants at the indicated ratios were injected. The mediastinal blocks were harvested 30 days later, and tumor growth was evaluated.

†Mean volume is calculated only for the tumors detected.

‡$P<.05$ compared to control groups by the Wilcoxon test.

§$P<.01$ for distribution of tumor incidence by Fisher's exact test.

potentially reverse the malignant phenotype of lung cancer cells with inactive p53.

For optimal growth and progression of tumors arising from human cancer cells in vivo, organ-specific tumor implantation is critical. Intratracheal injection of human lung tumor cells into nu/nu mice induces a pattern of tumor growth similar to that observed in human lung cancer patients (*20*). H226Br cells were successfully grown in the pulmonary environment and formed endobronchial tumors. We observed that the orthotopic growth of 4-day established H226Br tumors was significantly inhibited by the intratracheal administration of LNp53B retroviral supernatant in a dose-dependent fashion.

A few small tumors were observed in mice treated with the LNp53B retrovirus. It is possible that some tumor cells escaped retroviral infection and formed tumors that were small because there were few residual cells. Alternatively, some tumor cells that were infected with the wt-p53 virus continued to grow more slowly than the uninfected cells and thus formed small tumors. The detection of the p53 DNA sequence in the resultant tumor favors the latter explanation. Transduction of wild-type p53 by a retroviral vector can mediate apoptosis in some cell types but may also become stably integrated in cells, which then have a reduced rate of proliferation (*11*,*12*). Variations in the site of proviral integration and the level of wild-type p53 expression may influence the outcome of its effect on the cell.

Although the use of a retroviral vector favors integration in rapidly dividing tumor cells, it is unlikely that all or even most tumor cells take up the virus. This observation suggests that a bystander effect is operative with LNp53B-transduced H226Br cells inhibiting the growth of nontransduced H226Br cells. A bystander effect for wild-type p53-transduced lung cancer cells in culture has been previously demonstrated in experiments mixing wild-type p53-transduced and nontransduced cells (*11*). The molecular basis of this bystander effect is under investigation.

We (*19*) previously reported that intratracheal instillation of anti-sense K-ras retroviral supernatant prevented the orthotopic growth of human H460a lung cancer cells, which have a codon 61 mutation of the K-ras oncogene. In another study (*12*), in situ PCR hybridization indicated that the wt-p53 retroviral vector is capable of multilayer penetration into the three-dimensional structure of multicellular tumor spheroids. Taken together, these results indicate that microscopic tumors established in the bronchial epithelium can be efficiently infected with a retroviral vector expressing therapeutic genes and that in situ retrovirus-mediated gene transfer may be a useful strategy for manipulating genetic abnormalities of cancer cells in vivo. Recent progress in the methodology of molecular genetics has made it possible to identify genetic lesions such as inactivation of tumor suppressor genes by mutations or deletions in premalignant lesions (*21*,*22*). Specific gene-replacement approaches based on the type of mutation found in the target cancer may be useful as an adjunct to conventional therapies that improve the prognosis of NSCLC patients and may lead to the development of gene-specific prevention strategies.

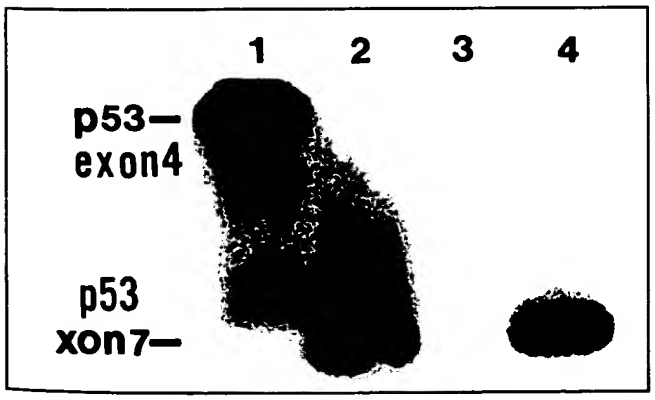

Fig. 3. Southern blot showing presence of wt-p53 in H226Br tumor DNA from nu/nu mouse 1 month after injection with either LNp53B or LNSX retroviral vectors. Lane 1, exogenous p53 PCR product (β-actin primer and p53 exon 4 primer) from H226Br-LNp53B (H226Br tumor DNA after treatment with LNp53B retroviral supernatant); lane 2, endogenous p53 PCR product (p53 exon 7 primers) from H226Br-LNp53B; lane 3, absence of exogenous p53 amplified by β-actin and p53 exon 4 primers from H226Br-LNSX (H226Br tumor DNA after treatment with LNSX retroviral supernatant); lane 4, endogenous p53 amplified by p53 exon 7 primers from H226Br-LNSX. A 2.3-kb p53 cDNA *Bam*HI fragment was used as a probe.

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Notes

Partially supported by Public Health Service grants R01CA45187, CA16672, and CA09611 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services; by gifts to the Division of Surgery from Tenneco and Exxon for the Core Laboratory Facility; and by a grant from the Mathers Foundation.

Manuscript received February 22, 1994; revised June 20, 1994; accepted July 13, 1994.

Activity of DMP 840, a New Bis-naphthalimide, on Primary Human Tumor Colony-Forming Units

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Background: **DMP 840 {(R,R)-2,2′-[1,2-ethanediylbis[imino(1-methyl-2,1-ethanediyl)]]-bis[5-nitro-1H-benz[de]-iso[quinoline-1,3(2H)-dione]dimethanesulfonate; NSC-D640430} is one in a series of bis-naphthalimides that binds DNA with high affinity and has sequence specificity to multiple G and C bases. It is also a potent inhibitor of RNA synthesis. DMP 840 has been selected for clinical evaluation on the basis of a broad spectrum of activity (including cures) in human tumors in murine models.** ***Purpose:*** **We evaluated DMP 840 in a human tumor clonogenic assay to estimate what plasma concentrations may be necessary for clinical cytotoxic activity and to determine what types of tumors potentially might be primary targets for initial phase II studies.** ***Methods:*** **A soft-agar cloning system assay was used to determine the in vitro effects of DMP 840 against cells from biopsy specimens of colorectal, breast, lung, ovarian, renal cell, stomach, and bladder cancers and from other tumor types. A total of 260 human tumor specimens were exposed continuously during the assay to DMP 840; 103 were assessable (20 colonies or more on control plates and 30% or less survival for the positive control). An in vitro response was defined as at least a 50% decrease in tumor colony formation resulting from drug exposure compared with controls.** ***Results:*** **In vitro responses were seen in 10% (one of 10), 54% (55 of 101), 80% (82 of 103), and 89% (82 of 92) of specimens tested at 0.01, 0.1, 1.0, and 10.0 μg/mL of DMP 840, respectively. At a concentration of 0.1 μg/mL, specific activity was seen against melanoma (80%) and against renal cell (80%), ovarian (63%), breast (54%), non–small-cell lung (42%), and colorectal cancers (33%). DMP 840 demonstrated activity in tumor specimens resistant in vitro to methotrexate (88%), doxorubicin (58%), platinum (57%), cyclophosphamide (53%), vinblastine (53%), etoposide (53%), fluorouracil (37%), and paclitaxel (36%).** ***Conclusions:*** **At in vitro concentrations of 0.1 μg/mL as a continuous exposure, DMP 840 has activity against a variety of human tumors, including a subgroup resistant in vitro to standard antineoplastic agents.** ***Implications:*** **Further clinical development of DMP 840 is warranted. [J Natl Cancer Inst 86:1462-1465, 1994]**

DMP 840 {(R,R)-2,2′-[1,2-ethanediylbis[imino(1-methyl-2,1-ethanediyl)]]-bis[5-nitro-1H-benz[de]iso[quinoline-1,3(2H-dione] dimethanesulfonate; NSC-D640430} (Fig. 1) is one in a series of bis-naphthalimides that binds DNA with high affinity and has se-

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See "Notes" section following "References."

Retrovirus-mediated wild-type *p53* gene transfer to tumors of patients with lung cancer

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A retroviral vector containing the wild-type *p53* gene under control of a β-actin promoter was produced to mediate transfer of wild-type *p53* into human non-small cell lung cancers by direct injection. Nine patients whose conventional treatments failed were entered into the study. No clinically significant vector-related toxic effects were noted up to five months after treatment. *In situ* hybridization and DNA polymerase chain reaction showed vector-*p53* sequences in posttreatment biopsies. Apoptosis (programmed cell death) was more frequent in posttreatment biopsies than in pretreatment biopsies. Tumor regression was noted in three patients, and tumor growth stabilized in three other patients.

Transformation of the normal cell to a malignant cell is causally related to the acquisition of a series of genetic lesions. Most of the currently known lesions cause either a gain of transforming function or loss of tumor suppressor function by the gene product. Despite the multiplicity of these lesions within a single cancer cell, studies have shown that correction of a single critical genetic lesion is sufficient to abrogate tumorigenicity in human cancer cells[1-3]. This raises the possibility that correction of a single genetic abnormality in the cancer cell could mediate a therapeutic effect. Two gene families that contribute to carcinogenesis are dominant oncogenes and tumor suppressor genes[4,5]. Tumor suppressor genes may undergo homozygous loss of function by mutation, deletion, methylation, or a combination of these. We postulated that it may be possible to directly restore expression of the *p53* tumor suppressor gene and that this could suppress tumor growth *in vivo*.

The existence of tumor suppressor genes was originally hypothesized to explain the inheritance pattern of retinoblastoma[6]. It was postulated that both copies of the putative tumor suppressor gene must be eliminated or inactivated to eradicate the growth-suppressive function of the gene[4]. Theoretically, replacement of a nonfunctioning copy of the tumor suppressor gene in cells with loss of function by a functioning copy could restore normal growth and proliferation pathways. Possible mechanisms by which such a replacement could cause clinically beneficial effects include induction of tumor cell death by direct killing (for example, apoptosis) or bystander effects (killing or growth arrest of nontransduced tumor cells mediated by transduced tumor cells), induction of tumor cell dormancy, or prevention of malignant progression in premalignant cells[7]. The *p53* gene is frequently rendered nonfunctional in human cancers by mutation or deletion and is therefore a candidate gene for replacement[8].

A retroviral expression vector was constructed to mediate efficient transduction of the wild-type *p53* gene into human lung cancer cell lines so the effects of wild-type *p53* expression in lung cancer could be studied in clinically relevant model systems of human lung cancer. A wild-type *p53* cDNA driven by a β-actin promoter was inserted into a modified LNSX retroviral vector[7]. Restoration of the wild-type *p53* gene suppressed growth of lung cancer cell lines and growth of human lung cancers in an orthotopic *nu/nu* mouse model, thus confirming previous observations that correction of a single genetic defect in the cancer cell decreases tumorigenicity[1,9]. These studies provided the basis for a clinical trial to test the safety and antitumor efficacy of retrovirus-mediated delivery of the wild-type *p53* gene to human lung cancer cells. Retroviral supernatant was directly injected into lung cancers with documented *p53* mutations by using either a fiberoptic bronchoscope or percutaneous needle with radiologic guidance.

Patient characteristics

Nine male patients, median age 68 years (range, 51–73), who had a history of primary non-small cell lung carcinoma (NSCLC) were entered into the study (Table 1). Four patients with recurrent endobronchial lesions (three squamous cell carcinomas and one adenocarcinoma) were treated with bronchoscopic injections of the retroviral *p53* expression vector ITRp53A. Four patients with chest wall lesions (two large cell carcinomas, one squamous cell carcinoma, and one adenocarcinoma) were treated with percutaneous injections under computed tomographic (CT, three patients) or fluoroscopic (one patient) guidance. One patient had

Table 1 Characteristics of patients and response to injections of retroviral vector ITRp53A

Pt no.	Sex	Age	Performance status (Zubrod)	Histologic	Prior treatme..:	Site of treatment	Route of treatment	Mutation (codon, base change, amino acid change)	Response of treated lesion (response duration in weeks)	Survival after treatment* (weeks)
1	M	60	1	squamous	drainage of pleural abscess, 50 Gy lung tumor, 15 Gy brachytherapy	left mainstem bronchus	bronchoscopic	138, CTC→CTG Leu→Val	viable tumor in pretreatment biopsy; no viable tumor at treated site by bronchoscopy, biopsy, and autopsy (17)	17
2	M	58	1	squamous	66 Gy lung tumor	right upper lobe	bronchoscopic	248, CGG→CTG Arg→Leu	viable tumor in pretreatment biopsy; no viable tumor in 6 posttreatment biopsies at 1 month (10)	22
3	M	61	1	large cell	surgical resection, 60 Gy post-op	right upper chest wall	percutaneous by CT	245, GGC→AGC Gly→Ser	stable by chest radiograph and CT scan (9); viable tumor in pretreatment biopsy; 3 posttreatment biopsies show no viable tumor	9
4	M	73	2	adenocar-cinoma	vinblastine, mitomycin, 2 months	left anterior chest wall	percutaneous by fluoroscopy	249, AGG→ATG Arg→Met	inevaluable	3
5	M	56	2	adenocar-cinoma	resection of solitary brain metastasis with whole-brain radiation, vinblastine, mitomycin, methotrexate, 6 months; paclitaxel, 2 months; 30 Gy to lung tumor	right upper lobe	bronchoscopic	269, frame-shift insertion	>50% regression of treated endobronchial tumor with viable tumor in pre- and posttreatment biopsies (4)	4
6	M	72	1	squamous	resection of brain metastasis with whole-brain radiation, paclitaxel, 3 months; 45 Gy to lung tumor 9 months before entry	right posterior chest wall	percutaneous by CT	157, GTC→TTC Val→Phe	progression by CT scan; viable tumor in pre- and posttreatment biopsies	13
7	M	57	1	large cell	cisplatin, VP-16, 5-FU, 6 months; surgical resection, 63 Gy post-op; docetaxol, 2 months	left adrenal metastasis	percutaneous by CT	135, TGC→TAC, Cys→Tyr	stable with increased lucency on CT scan suggestive of tumor necrosis and relief of flank pain (8); viable tumor in pre- and posttreatment biopsies	22
8	M	51	1	large cell	surgical resection; ifosfamide, mitomycin & cisplatin, 9 months; 50 Gy chest wall radiation 5 years before entry	left posterior chest wall	percutaneous by CT	150, ACA→ATA, Thr→Ile 157, GTC→TTC, Val→Phe 175, CGC→ AGC, Arg→Ser	stable(8); viable tumor in pre- and posttreatment biopsies	20
9	M	68	2	squamous	52 Gy lung tumor, 20 Gy right bronchus, and 6.7 Gy at 10 mm left bronchus	carina	bronchoscopic	145, TGG→TGA, Trp→stop	inevaluable	6

*All patients died from progression of untreated metastases or other complications.

a left adrenal metastasis from a large cell carcinoma treated with percutaneous injection under CT guidance. All patients in the study had recurrent or metastatic tumors that had progressed during prior treatment. Five patients had undergone surgical resection of either the primary lung cancer (three patients) or a brain metastasis (two). Five patients had received chemotherapy and eight patients had received radiation therapy.

Detection of vector

Polymerase chain reaction (PCR) analysis using primers specific for the retroviral transgene was used to detect the presence of vector sequences in tumor biopsies. PCR did not detect a retroviral sequence in any of the pretreatment biopsies. DNA extracted during posttreatment biopsies from two patients and post-mortem tumor from one patient showed the presence of vector-derived sequences by PCR (Table 2 and Fig. 1). Six patients showed nuclear localization of the vector by *in situ* hybridization using the *neo*' probe (Table 2 and Fig. 2). Thus all patients, with the exception of patient 9, who did not complete the treatment protocol, had evidence of gene transfer.

TUNEL staining of treated tumor specimens

Pretreatment and posttreatment specimens suitable for terminal deoxynucleotidyl transferase (TDT)-mediated biotin dUTP nick end-labeling (TUNEL) staining were available from seven patients. Six of the seven patients had increased TUNEL staining in posttreatment biopsies compared to the pretreatment baseline levels (Table 2 and Fig. 3).

Effects on tumor growth

Seven of the nine patients were evaluable for effects of the injection on tumor growth. Two patients were not evaluable for response, one because he died before the end of the 1-month evaluation period and one because he was unable to complete the course of treatment. Patient 4 developed aspiration pneumonia after placement of a percutaneous gastrostomy tube and died 3 weeks after the first retroviral dose. Patient 9 developed muscle weakness and respiratory distress secondary to administration

Table 2 Assessment of gene transfer by ITRp53A retroviral vector

Pt no.	DNA-PCR (score)	*In situ* hybridization[a] (%)	TUNEL staining[b]
1	+	3	17.0
2	–	3	34.0
3	+	ND[c]	ND
4	+	ND	27.6
5	–	2	10.2
6	–	2	10.2
7	–	2	0.8
8	–	1	23.0
9	ND	ND	ND

Results of the indicated assays on posttreatment biopsy specimens are shown. No pretreatment samples were positive in DNA-PCR and *in situ* hybridization assays. Examples are shown in Fig. 1–3.

[a]All sides were coded and read blinded by a single observer who had no knowledge of the patient or collection date. The percentage of tumor cells with punctate nuclear staining was determined in 500 cells per slide (×400 magnification). The slides were evaluated by the following scoring system: 0, no staining; 1, <5% of the cells stained; 2, 5 to 20% of the cells stained; 3, >20% of the cells stained. Slides staining ≥1 were considered positive; the maximum score for each patient is given; significant increases in *in situ* hybridization were observed in 6 of 6 evaluable cases ($P < 0.05$, sign test).

[b]Cells with morphologic features of necrosis were not included among the TUNEL-positive cells. The percentage of cells with nuclear staining was determined in 500 cells per slide (×400 magnification). The background level of TDT staining in pretreatment samples was <4%. All sides were coded and read blinded by a single observer who had no knowledge of the patient or collection date. The maximum percent of cells staining positively is given for each patient. Mean percent ± s.d. of TUNEL-stained cells: 1.0 ± 0.5 for all pretreatment biopsies; 10.6 ± 2.9 for posttreatment biopsies. Six of seven biopsies showed definitive increases in TUNEL staining after treatment ($P < 0.05$, two-sided Wilcoxon signed rank test).

[c]ND, not done because of an unsatisfactory or unavailable specimen.

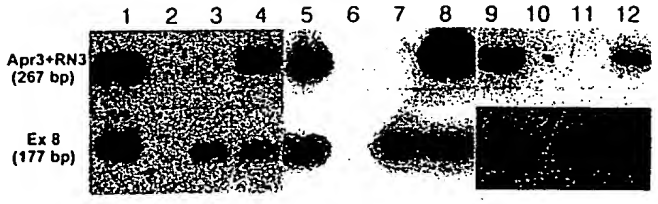

Fig. 1 Polymerase chain reaction evaluation of vector sequences in DNA extracted from biopsies of injected tumors from patients 1 (lanes 1–4) and 3 (lanes 9–12) and an autopsy specimen of an injected tumor from patient 4 (lanes 5–8). PCR was performed with either the Apr3 + RN3 primers specific for ITRp53A sequences or *p53* exon 8 primers that recognize endogenous *p53* sequences as a positive control. Lanes 1, 5, 9: ITRp53A plasmid control; lanes 2, 6, 10: primer only control; lanes 3, 7, 11: pretreatment biopsy; lanes 4, 8, 12: posttreatment tumor specimen day 1, day 18 and day 5. The PCR products were probed with a ^{32}P-labeled, nick-translated *p53* cDNA *Bam*HI fragment.

a general anesthetic following initial bronchoscopy, and treatments were discontinued. The patient required respirator support for 5 days and ultimately recovered.

Three of the seven patients showed evidence of tumor regression in the treated lesion. Details of the cases are as follows:

(1) Patient 1 had recurrence of a squamous carcinoma in the left mainstem bronchus at the bifurcation of the left upper and lower lobes. Bronchoscopy 1 month after retroviral injection showed marked regression of the tumor mass, and two biopsies were negative for viable tumor (Fig. 4). Three bronchoscopic biopsies in the region of the original tumor after 3 months showed no evidence of viable tumor. The patient died 4 months after treatment secondary to tumor progression at untreated sites in the left lung and distant metastases. Autopsy revealed no evidence of invasive cancer in the epithelium of the treated left mainstem bronchus.

(2) Patient 2 had an endobronchial recurrence obstructing the right upper lobe after radiation therapy. Six separate biopsies 1 month after retroviral injection showed no evidence of viable tumor at the right upper lobe orifice (Fig. 5). The patient developed progressive nodal disease in the mediastinum 2 months later.

(3) Patient 5 had tumor remaining after radiation therapy obstructing the right upper lobe orifice. Six weeks after completion of radiation therapy the patient received a single cycle of five daily endobronchial injections of the *p53* retroviral vector into the tumor mass. The patient died 1 month later from distant progression and pneumonia. At autopsy, the right upper lobe orifice was patent, with >50% regression of the treated endobronchial tumor (Fig. 4).

Patient 3 had radiographic stabilization of disease for 9 weeks following a single treatment cycle. The patient died of complications related to resection of a progressively enlarging cervical vertebral body metastasis. Patient 8 had stabilization of an injected chest wall lesion for 8 weeks after two treatment cycles before a second untreated intrathoracic tumor progressed. Patient 7, after two treatment cycles, had stabilization of an adrenal metastasis for 8 weeks before the lesion progressed. In this patient, a CT scan 2 weeks after injection revealed increased lucency at the injection site, suggesting nonviable tumor representing 30% of the volume of the mass. During this time a smaller right adrenal metastasis enlarged progressively. Patient 6 had a chest wall lesion which continued to progress after the first cycle of treatment. Each of these patients had other sites of disease not treated by gene replacement injection that continued to progress during treatment with the vector. In each patient, progression at untreated sites was evident at each monthly evaluation, contrasting with the stabilization or regression noted in the treated lesions.

The degree of inflammatory cell infiltrate showed no consistent changes from the pretreatment biopsies to the biopsies taken after injection. Western blot analysis of the posttreatment serum from six of the patients did not show the presence of antibodies to retroviral proteins (data not shown).

Toxicity

There were no toxic effects directly attributable to the vector. Three patients had complications related to the procedures involved in administering the vector. Patient 1, who was treated under general anesthesia, had transient fever and required intubation for several hours after the first bronchoscopy. Subsequent procedures were done under topical anesthesia without complications. Patient 7 had a pneumothorax during CT-guided

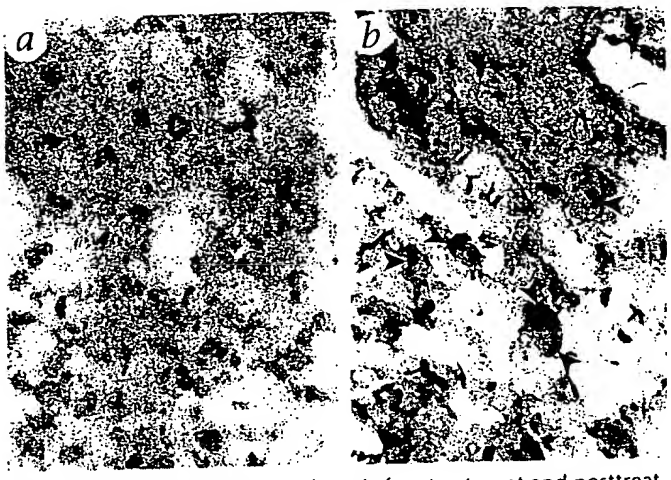

Fig. 2 *In situ* hybridization with *neo*' of pretreatment and posttreatment tumor biopsies from patient 1. *a*, Pretreatment biopsy; *b*, 24-h posttreatment biopsy; probe: LNSX plasmid. Arrows point to examples of punctate nuclear staining. Additional negative controls not shown include saline and pBR plasmid. Magnification, ×1000.

injection of an adrenal metastasis. The pneumothorax was small and did not require a thoracostomy tube. Patient 9 required intubation during the first bronchoscopic injection because of difficulty with ventilation. Subsequently, the patient required mechanical ventilation and prolonged intubation. It was believed that the patient's pulmonary function was not adequate to tolerate subsequent bronchoscopies, and the patient was removed from the protocol. He was extubated after 5 days and discharged from the hospital.

Safety

All patients had lymphocytes and sputum samples collected for up to 3 months posttreatment. Three patients (1, 4 and 5) underwent autopsy at 17, 3 and 4 weeks following entry into the protocol. Primers specific for vector sequences were used in the PCR analysis of DNA extracted from lymphocytes and sputa of patients before, during and after treatment. None of the nontumor tissues analyzed by PCR, including lymphocytes, tracheal mucosa, brain, uninjected lung, gastrointestinal tract tissues, skeletal muscle, heart, spleen, liver and testes, showed retroviral sequences, although the tissues had intact DNA as shown by amplification of endogenous *p53* exon 8 in each sample and retroviral sequences from the injected tumor of patient 4 (data not shown).

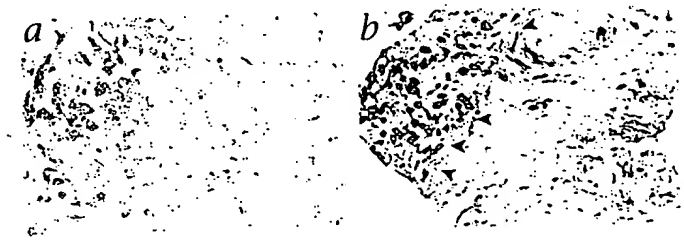

Fig. 3 Staining by TUNEL technique of pretreatment (*a*) and 24-h posttreatment (*b*) biopsies from patient 1. Additional positive and negative controls not shown include H1299 *p53* homozygously deleted human NSCLC tumors grown in *nu/nu* mice injected with adenoviral vector AdCMV-*p53*, which expresses wild-type *p53*, and all sections processed in the absence of TDT. Arrows outline region of positive nuclear staining. Magnification, ×400.

Discussion

Rationale and preclinical studies. Preclinical studies in *nu/nu* mice bearing tumors derived from human NSCLC cell lines showed that regional administration of viral vectors expressing wild-type *p53* prevents growth of tumors with *p53* mutations in orthotopic tumor models and mediates regression of large established tumors[9–11]. These studies indicate that retroviral gene transfer into tumors following direct injection may occur at levels sufficient to mediate clinically significant tumor regression. Retroviral gene transfer is more efficient in cancer cells than was anticipated from studies in normal tissues[12]. The selectivity of proviral integration events for proliferating cells favors integration by tumor cells. Fresh human cancers have a relatively high fraction of proliferating tumor cells[13–15]. Viral vectors spread readily through three-dimensional cancer cell matrices, as determined by studies with spheroids and in solid tumors in *nu/nu* mice[10,11].

The observation that correction of a single genetic lesion in human cancers with multiple genetic lesions can cause regression of the tumors was critical to the development of this therapeutic approach[1,9,10]. Restoration of wild-type gene function in a single critical pathway appears sufficient to initiate apoptosis or G_1 arrest, resulting in either tumor regression or inhibition of tumor growth.

Efficiency of gene transfer. The presence of the transgene was detected in some but not all tumors by DNA PCR, with the longest duration appearing in an autopsy specimen 3 weeks after treatment. *In situ* hybridization provided additional evidence for uptake and nuclear localization of the vector by tumor cells.

Although direct demonstration of gene expression was not documented by reverse transcriptase PCR (data not shown), the observation of an increase in TUNEL staining indicative of apoptosis in posttreatment tumor biopsies provides indirect evidence for gene expression. Expression of wild-type *p53* in tumor cells with mutant *p53* can mediate apoptosis[16,18,19]. The increase in apoptosis seen in posttreatment biopsy specimens suggested that the expression of wild-type *p53* in tumors lacking *p53* function can mediate apoptosis in human tumors *in vivo*, as has been shown *in vitro* and in animal models[11,16,18,20–22]. The rapid response of some of the tumors is not surprising, as apoptotic cell death is observed in lung cancer cells in culture within 24 hours of wild-type *p53* expression (T. Timmons and J.A.R., unpublished observations). It is also possible that some of the effects seen were the result of G_1 arrest causing a decrease in the proliferation rate of the remaining tumor cells[23–27].

There are several possible explanations for our inability to detect retroviral sequences in all tumor biopsies. The high degree of cancer cell death in the posttreatment biopsies, and thus the absence of viable cells, may explain our inability to identify intact retroviral sequences in many of the tumor specimens. Other factors influencing detection of retroviral sequences in posttreatment biopsies include low levels of expression, induction of apoptosis, biopsies yielding low amounts of undegraded RNA, and the lower sensitivity of the reverse transcriptase PCR[28].

Although the efficiency of transduction of retroviruses into tumor cells is low compared to that of adenoviruses, our study indicates that it is sufficient to mediate a therapeutic effect. The *in situ* hybridization studies showed that in some regions of the tumor more than 20% of tumor cells had taken up the virus. The magnitude of the therapeutic responses seen suggests that bystander effects may have contributed to the tumor regressio

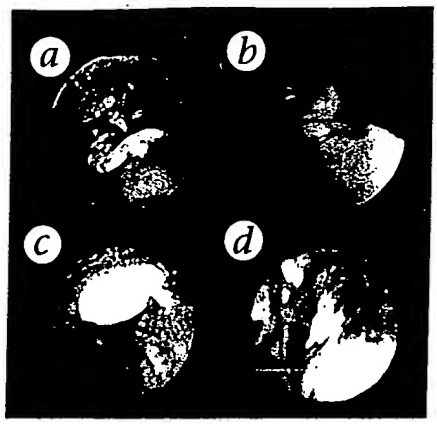

Fig. 4 Pretreatment (*a* and *c*) and 30-day posttreatment (*b* and *d*) bronchoscopic images of patients 1 (*a* and *b*) and 5 (*c* and *d*). *a*, The lesion is situated in the left mainstem bronchus on the division of the upper and lower lobes; biopsies showed squamous cell carcinoma (arrows). *b*, Left mainstem bronchus 30 days following ITRp53A injection; five independent biopsies showed absence of viable tumor cells. *c*, Adenocarcinoma obstructing the right upper lobe orifice. *d*, Right upper lobe orifice (arrows) 30 days following treatment; biopsies in this region showed residual adenocarcinoma.

observed. The bystander effect of the *p53* gene was first observed in mixing experiments of human NSCLC cells retrovirally transduced with the wild-type *p53* gene[7]. Additional supporting evidence for the existence of a bystander effect comes from *in vivo* studies, showing that the therapeutic effect of wild-type *p53* gene replacement exceeds that expected from the fraction of cells transduced by viral vectors[9]. For some patients, the degree of tumor regression exceeded that expected from the percentage of transduced cells, suggesting that a bystander effect occurred.

Lack of toxicity. The lack of toxicity attributable to the vector is encouraging and suggests that a wide window exists for these agents in which therapeutic benefit is not accompanied by additional toxicity. Grading of inflammatory response in the tumor showed no consistent changes following vector injection. Thus, it appears unlikely that an immunologic or nonspecific inflammatory response was the mechanism of tumor regression.

Potential for clinical application. Several potential clinical applications exist for this technology. Local control of many solid tumors remains suboptimal. For example, lung cancer patients who present with unresectable local tumors have a greater than 50% recurrence rate at the local site despite combined treatment with chemotherapy and radiation[29]. The lack of significant toxicity for the wild-type *p53* retroviral vector suggests that this agent could be applied more aggressively at the margin between tumor and normal tissue than existing local ablative treatments.

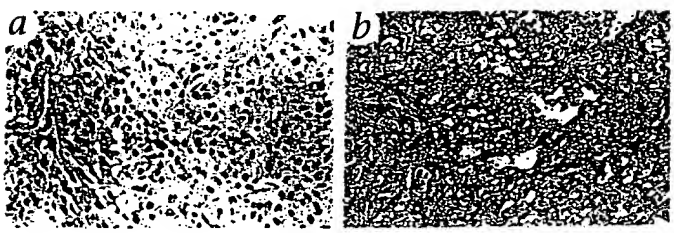

Fig. 5 Histologic sections of pretreatment (*a*) and 30-day posttreatment (*b*) biopsies from the right upper lobe of patient 2. *a*, Squamous cell carcinoma; *b*, no viable tumor cells. This is representative of six independent biopsies of the right upper lobe orifice taken at this time. Hematoxylin and eosin stain. Magnification, ×400.

Recent studies have indicated that expression of wild-type *p53* can be synergistic with DNA-damaging agents such as cisplatin and radiation therapy in inducing apoptosis in tumors[30]. Thus, the combination of *p53* as a neoadjuvant or adjuvant with these primary treatments may improve local tumor control for lesions that are not resectable or are nonresponsive to radiation or chemotherapy alone. It is also established that premalignant lesions such as Barrett's esophagus and bronchial dysplasias have oncogene and tumor suppressor gene mutations that precede the development of invasive cancers[31–35]. These lesions are localized and, conceivably, could be directly injected with therapeutic vector through an endoscope with the goal of preventing the development of invasive malignancy.

In this study tumor regression was noted only in endobronchial tumors and was limited to the local tumor. The authors plan to continue this study in endobronchial tumors but with three rather than five injections to determine whether the lower dose is efficacious. Future improvements in vector design and production techniques may increase the efficacy of retrovirus-mediated gene transduction and extend the clinical applications. A second series of clinical trials is now in progress using a replication-defective E1-deleted adenovirus to deliver wild-type *p53*, and this may achieve higher transduction efficiencies and higher levels of expression than the retroviral vector. Comparison of the efficacy and toxicity profiles of these vectors, as well as their use in combined modality protocols, will be of interest.

Methods

Protocol approval. This protocol was approved by the Biosafety and Surveillance Committees of the M.D. Anderson Cancer Center, the Recombinant DNA Advisory Committee of the National Institutes of Health, and the US Food and Drug Administration (FDA)[36,37].

Gene transfer vector. A 1.8-kb wild-type *p53* cDNA fragment linked to a β-actin promoter was subcloned into the LNSX retroviral vector[38] in 3' to 5' orientation following removal of an SV40 promoter contained in the original LNSX vector[7]. The new construct was called ITRp53A. Amphotropic packaging cell line GP+envAm12 (gift of A. Bank) was used. A single lot of the retroviral vector with a titer of 5×10^7 colony-forming units per milliliter was produced for the clinical trial at the M.D. Anderson Cancer Center and stored in 10-ml syringes at –80 °C. Protamine was added at a concentration of 5 μg/ml to enhance retroviral transduction efficiency[39].

Eligibility criteria. Nine patients with histologic proof of NSCLC entered the trial. Patients had unresectable tumors and were either unable to receive primary external beam radiation therapy or had had a recurrence after such therapy. Patients were also eligible if they did not respond to or relapsed after chemotherapy. Patients had either endobronchial tumor accessible by the bronchoscope with some clinical evidence of bronchial obstruction, advanced local-regional cancer which was unresectable, or isolated metastases whose regression or stabilization would offer potential benefit to the patient. Patients with central nervous system or gonadal tumors were excluded. All patients had a performance status of ≤2 as measured on the Zubrod scale. The patients signed an informed consent document indicating that they were aware of the investigational nature of this study, in keeping with the policies of the M.D. Anderson Cancer Center. Mutations in the *p53* gene were identified by single-strand conformation polymorphism (SSCP) analysis and

DNA sequencing of a tumor biopsy as described previously[40]. All mutations were confirmed by sequencing or SSCP analysis of a second independent PCR reaction. Patients were not treated on protocol until 4 weeks after completion of systemic or local therapy.

Treatment protocol. The treatment protocol and safety studies have been described previously[37]. Patients underwent flexible fiberoptic bronchoscopy under topical or general anesthesia. A Stifcor transbronchial aspiration needle (21 gauge) was passed through the biopsy channel of the bronchoscope. The tumor site was injected with 10 ml of the appropriate retroviral supernatant. For endobronchial lesions, the 10-ml volume was divided equally among a central intratumoral injection site and two to five peripheral injection sites. Tissue was biopsied and debrided with the 1-mm biopsy forceps. For chest wall tumors, two or three 22-gauge Chiba needles were placed, equally spaced, into the tumor, and the 10-ml volume was divided equally among these injection sites. Patient 7, with the adrenal metastasis, had a single needle placed at a different site in the tumor each day. A neodymium:yttrium-aluminum-garnet laser was used for surface coagulation during the first day of treatment for patient 1. Viable tumor was present in subsequent biopsies during treatment. Lasers were not used at any other time during the protocol. After patient 6 entered the study, the FDA granted permission to treat patients with multiple cycles. Patients with locoregional tumors received intratumoral vector injections at multiple sites percutaneously under fluoroscopic guidance for a total of five daily injections. The maximum volume was 10 ml per injection.

Response and toxicity. All patients were evaluable for response and toxicity following one course of therapy. The toxic effects of therapy were evaluated according to the National Cancer Institute's Common Toxicity Criteria[41]. Response to therapy was assessed by chest roentgenogram or CT scans before each course of treatment. Patients were evaluable for response if they had received at least one course of therapy followed by an appropriate radiograph to document response. A complete response was defined as disappearance of all clinical evidence of tumor in the treated area without the appearance of new lesions for a period of at least 4 weeks. Patients evaluable for a less-than-complete response were those who still had a bidimensionally measurable tumor. In cases for which serial CT scans were available, tumor responses were determined on the basis of volumetric measurements. All measurements were made by one radiologist (D.L.). Partial response was defined as a 50% or greater reduction in the sum of the products of the diameters of the measurable tumor; a minor response was defined as a 25% to less than 50% reduction in the sum of the products of the diameters of the measurable lesion. Patients were designated as having progressive disease if they showed a 25% or greater increase in the size of their tumor or if they developed unequivocal new lesions during treatment in the treated area; they were considered as having no change if the treated tumor changed in any way that did not meet the criteria described above. The time to progression was measured from the first observation of reduction in tumor bulk until there was evidence of progressive disease.

For endobronchial lesions, the tumor bed was photographed at a specified distance before each course of therapy. The longest diameter and its perpendicular were measured. Size was reported as the product of the diameters. Endoscopic tumors that were not measurable were considered evaluable for response based on a minimum of four biopsies of the treated tumor bed.

Survival duration was measured from time of entry into the protocol. Each patient's response was reviewed by a Data Management Committee consisting of a medical oncologist, radiation oncologist, and thoracic surgical oncologist.

Efficacy of gene transfer. PCR analyses were performed on pretreatment and posttreatment tumor and normal tissue biopsies, peripheral blood mononuclear cells, sputa, and specimens collected at autopsy as described previously[9]. A nested PCR was used to amplify proviral sequences. The primers amplified junction sequences between *p53* exon 3 and the β-actin promoter. The first set of primers was Rev Ex3 (5'–CAA ATC ATC CAT TGC TTG GGAC–3') and Apr2 (5'–GAC TCT AGC TGC GAG AAT AGG–3'). The second set of primers was RN3 (5'–GGG GAC AGA ACG TTG TTT TCG–3') and Apr3 (5'–TGG GCT GCA GGT CGA CTC TAT–3'). Exon 8 of *p53* was amplified as an internal control for the presence of DNA using primers Ex8A (5'–TTG GGA GTA GAT GGA GCC TT–3') and Ex8B (5'–ACA GAG GAA GAG AAT CTC CG–3'). Thirty-five amplification cycles were performed for each set of primers. The PCR analysis had a sensitivity of one copy in a background of 10^5 genomes.

***In situ* hybridization.** A *neo* gene probe was used to avoid the cross-hybridization of a *p53* probe with endogenous *p53* sequences. Slides were hybridized at 42 °C overnight with the digoxigenin-labeled LNSX plasmid. Negative controls for each section included saline and pBR plasmid. A positive control, human H460 NSCLC cells infected with LNSX at the single-copy level grown in *nu/nu* mice as a subcutaneous tumor, was included in each experiment. The slides were incubated with 0.2% anti-digoxigenin-alkaline phosphatase-conjugated antibody in 100 mM Tris-HCl buffer (pH 7.5). The color reaction was developed with a phosphate and polyvinyl alcohol buffer. The slides were counterstained with Giemsa stain.

TUNEL assay for DNA fragmentation. The TUNEL assay was a modification of a previously described technique[16,22]. Slides were counterstained with 0.4% methylene green. Corresponding hematoxylin and eosin-stained slides were evaluated for the presence of an inflammatory cell infiltrate and were graded on a scale of 1 to 4.

Statistical analysis. A single-arm study design was used. To prevent enrolling more patients than necessary in the trial if excessive toxicity was found, a Bayesian early stopping rule was implemented. The Wilcoxon signed-rank test and the sign test were used for comparisons before and after treatment of the percentages of cells showing TUNEL staining and *in situ* hybridization, respectively.

Acknowledgments

We thank Bert Vogelstein and Curt Harris for reviewing the manuscript. We thank the patients who participated in this study and their families; B. Bready, L. Trissel, R. Anderson and the Division of Pharmacy, the University of Texas M.D. Anderson Cancer Center; E. Janssen for monitoring biosafety; C. Torrence, T. Hays, and C. Brunelli for their assistance in preparing and maintaining the RAC and FDA protocol submissions; and M. Contreras and K. Hale for preparation of the manuscript. The University of Texas and J.A.R. have received an equity interest in Introgen Therapeutics, Inc. The terms of this agreement have been reviewed and approved by the M.D. Anderson Cancer Center in accordance with its conflict-of-interest policies. This work was funded by a grant from the National Cancer Institute (R01 CA 45187) (J.A.R.), by gifts to the Division of Surgery from Tenneco and Exxon for the Core Lab Facility, by the M.D. Anderson Cancer Center Support Core Grant (CA 16672), and by a sponsored research agreement from Introgen Therapeutics, Inc. (Austin, Texas, 78701). T.J.M. is a Pew Scholar in the Biomedical Sciences.

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Effects of ligand activation of peroxisome proliferator-activated receptor γ in human prostate cancer

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Communicated by Baruj Benacerraf, Dana-Farber Cancer Institute, Boston, MA, July 14, 2000 (received for review December 9, 1999)

Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear hormone receptor that plays a key role in the differentiation of adipocytes. Activation of this receptor in liposarcomas and breast and colon cancer cells also induces cell growth inhibition and differentiation. In the present study, we show that PPARγ is expressed in human prostate adenocarcinomas and cell lines derived from these tumors. Activation of this receptor with specific ligands exerts an inhibitory effect on the growth of prostate cancer cell lines. Further, we show that prostate cancer and cell lines do not have intragenic mutations in the PPARγ gene, although 40% of the informative tumors have hemizygous deletions of this gene. Based on our preclinical data, we conducted a phase II clinical study in patients with advanced prostate cancer using troglitazone, a PPARγ ligand used for the treatment of type 2 diabetes. Forty-one men with histologically confirmed prostate cancer and no symptomatic metastatic disease were treated orally with troglitazone. An unexpectedly high incidence of prolonged stabilization of prostate-specific antigen was seen in patients treated with troglitazone. In addition, one patient had a dramatic decrease in serum prostate-specific antigen to nearly undetectable levels. These data suggest that PPARγ may serve as a biological modifier in human prostate cancer and its therapeutic potential in this disease should be further investigated.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily that includes thyroid hormone, retinoic acid, and androgen and estrogen receptors. These receptors share common features, including a central DNA binding domain and a carboxyl-terminal domain responsible for dimerization, ligand binding, and transcriptional activation. PPARγ requires heterodimerization with the retinoid X receptor for binding to DNA at specific sites, defined as direct repeats of hormone response elements, separated by one base (DR-1).

PPARγ transcriptional activity depends on the binding of ligands. Although the identity of a true endogenous ligand for PPARγ is still unclear, both natural and synthetic ligands have been described. Natural ligands, such as certain polyunsaturated fatty acids and 15-deoxy$\Delta^{12,14}$ prostaglandin J_2, have been shown to bind to this receptor at concentrations in the micromolar range (1–3), whereas the synthetic antidiabetic thiazoledinediones are able to bind to PPARγ with a K_D of 50–700 nM (2, 4).

There are several thiazoledinedione ligands for PPARγ, including troglitazone (Rezulin), rosiglitazone (Avandia), and pioglitazone (Actos). The latter two currently are used in the treatment of type 2 diabetes mellitus. In the last 2 years, troglitazone has been prescribed for more than one million patients in the United States alone (5). These drugs improve insulin resistance, and troglitazone has been shown to be effective at doses ranging between 400 and 800 mg/day. Although the drug generally has been well tolerated at these doses, idiosyncratic liver toxicity has been noted in some patients (6). Thiazoledinediones likely work in the context of type 2 diabetes through the activation of PPARγ in adipose and/or muscle cells, but the downstream target genes that are relevant to the insulin-sensitizing effects of these drugs are still unknown.

PPARγ is expressed at highest levels in adipose tissue (7, 8). Its dominant role in the differentiation of this tissue was elucidated through experiments using exogenous expression and ligand activation in several fibroblastic cell lines. In this context, PPARγ was shown to induce the morphology and the pattern of gene expression characteristic of terminally differentiated adipocytes (9). More recently, it has been shown that PPARγ is expressed at significant levels in nonadipose cells, including epithelial cells (10–14). Its physiological function in those tissues remains unknown, although activation seems to induce certain characteristics of differentiation appropriate for those cell types.

Ligand activation of this receptor causes growth arrest in several cell types derived from tumors (10–15). Recently, we also have demonstrated that the administration of troglitazone to patients with liposarcoma dramatically enhances adipocytic differentiation, including increased expression of the genes of terminal cell differentiation and a reduction in expression of Ki-67, a histochemical marker for cell proliferation *in vivo* (16). This is a striking example of induction of differentiation of solid tumors in humans.

We recently have found naturally occurring somatic mutations in the gene encoding PPARγ in a proportion of sporadic colorectal carcinomas (17). Each of the mutations causes a profound loss of function on this receptor. One mutation causes a truncation before the ligand binding domain whereas the others result in mutant receptors that do not bind to either natural or synthetic ligands. These findings suggest that PPARγ behaves like a tumor suppressor and that loss-of-function mutations might be etiologic for colorectal carcinogenesis. More importantly, our observations might suggest that examining for the presence of PPARγ mutations before institution of ligand differentiation therapy might be useful to predict whether such therapy would result in a response.

Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ; PSA, prostate-specific antigen; PGJ_2, prostaglandin J_2.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.180329197. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.180329197

Prostate cancer is the most common malignancy in American men. In 2000, there will be approximately 180,400 new prostate cancer cases and 31,900 prostate cancer deaths (18). Despite advances in early detection/treatment and management of advanced disease, the annual number of prostate cancer deaths has been relatively stable over the past decade. Improvements in the quality and length of life for men with prostate cancer will require the identification of novel treatment strategies.

In this study we show that PPARγ is expressed in normal prostate tissue and prostatic adenocarcinomas. Ligand activation of this nuclear hormone receptor induces growth inhibition in prostate cell lines. We also report the findings of a prospective clinical study of 41 patients with advanced prostate cancer, who received the PPARγ ligand troglitazone. Furthermore, to determine whether in prostate cancers, like in colon cancers, PPARγ mutations might render the tumor unresponsive to ligands, we determined the mutation status of this gene in cell lines and in a series of 38 prostate carcinomas.

Materials and Methods

Tissue Samples and Cell Lines. The human prostate normal-tumor pairs were obtained from the Brigham and Women's Hospital, Department of Pathology. The tissues for RNA analysis were resected from patients with primary medium grade tumors and frozen in liquid nitrogen. The human prostate cancer cell lines LNCaP, DU145, and PC3 were obtained from the American Type Culture Collection. DU145 cells were cultured in DMEM, PC3 cells in Ham's F-12 medium, and LNCaP cells in RPMI 1640. All media were supplemented with 10% FCS (HyClone).

RNA, Protein, and Cell Growth Analysis. Cells were grown in their respective media and, when confluent, harvested for Northern or Western blot analysis. Total RNA was extracted from grinded tissues and from cell lines with Triazol (GIBCO), according to manufacturer instructions. Twenty five micrograms of total RNA was loaded on a formaldehyde gel and subsequently transferred on a nylon membrane (Biotrans, ICN). Hybridization was performed by using a human PPARγ cDNA probe. For Western blot, cells were lysed according to protocols described previously (19). One hundred fifty micrograms of protein was loaded on a 10% urea-acrylamide gel. For growth assays, 1×10^3 cells were plated per well, in 24-well plates. After 48 h, DU145 cells were treated with different doses of troglitazone, or rosiglitazone (5 μM), 15-deoxy-$\Delta^{12,14}PGJ_2$ (5 μM), M2 (10 μM), Wyeth (10 μM), or GW0233 (5 μM). PC3 and LNCaP cells were treated with troglitazone at 10 μM. The cells were maintained in medium with 10% charcoal-stripped serum, and the medium containing the drugs was replaced every 2–3 days. Cells were counted with a Coulter Z1 counter at the times indicated.

Quantification of Prostate-Specific Antigen (PSA) Secretion. LNCaP cells at passage 25 were plated at a density of 3×10^3 cells per well, into 12-well plates and allowed to attach for 48 h. Cells were treated with 1 ml of freshly diluted drug or culture medium daily. Every 24 h, the supernatants were collected and the adherent cells were trypsinized and counted with a Coulter Z1 counter. The amount of PSA secreted was measured by using the Tandem-E PSA assay (Hybritech) according to the company's instructions.

Patients. Forty-one patients were enrolled in this study. All patients had histologically confirmed adenocarcinoma of the prostate and the disease progression, after local therapy or androgen deprivation, was defined as either: (*i*) rising PSA >1 ng/ml and >150% the nadir PSA after radical prostatectomy, (*ii*) rising PSA >4 ng/ml and 150% the nadir PSA after prostate radiation therapy, or (*iii*) rising PSA >150% nadir PSA after androgen deprivation and antiandrogen withdrawal. Exclusion criteria included symptomatic metastatic disease, radiation therapy within 28 days, antiandrogens or glucocorticoids within 4 weeks, cancer and leukemia group B performance status >2, or aspartate amino transferase (AST) >1.5 times the upper limit of normal. The protocol was approved by the Institutional Review Board and all patients provided written informed consent before study entry.

Pretreatment evaluation included history and physical examination, determination of performance status, alanine amino transferase (ALT), and PSA. Radiographic staging was not required. Troglitazone was provided as 400 mg tablets. Patients were instructed to take two tablets (800 mg/day) by mouth daily. A minimum of 12 weeks of treatment was planned, but troglitazone was continued for >12 weeks at the discretion of the treating physician. Toxicity assessment was performed every 4 weeks by using National Cancer Institute common toxicity criteria. Evaluations occurred monthly at which time PSA levels and liver function tests were obtained. All patients were asymptomatic. No patient had bidimensionally measurable disease at study entry. Treatment was discontinued in all patients in April 2000 after the Food and Drug Administration recommended withdrawal of troglitazone from the market because of liver toxicity.

Mutation Analysis. Genomic DNA was extracted from paraffin-embedded tissue sections of primary human prostate carcinomas as well as from adjacent nontumor tissue, by using standard protocols (20). The genomic DNA was used as template for PCR-based mutation analysis. Primers used for denaturant gradient gel electrophoresis span all seven coding exons of the gene, exon-intron junctions, and flanking intronic sequences. The seventh exon (exon II), which lies upstream of exon 1, represents the splice variant PPARγ2. Mutation analysis was carried out in four multiplex groups comprising a total of 14 fragments as described (21).

To determine whether PPARγ was deleted, microsatellite marker D3S1263, which is either in the gene or no more than 1 Mb from the gene, was used for loss of heterozygosity analysis, as described (22).

Results

PPARγ Is Expressed in Human Prostate Adenocarcinomas and Cell Lines. To determine whether PPARγ is expressed in human prostate tissue, we analyzed PPARγ mRNA levels in normal and prostatic adenocarcinomas obtained from five patients. As shown in Fig. 1A, PPARγ is expressed in both normal and malignant tissue obtained from the same patients. Its levels appear to be somewhat reduced in the tumors, compared with normal tissue. We subsequently analyzed PPARγ levels in the androgen-sensitive cell line LNCaP, and two androgen-independent cell lines, DU145 and PC3. As shown in Fig. 1*B*, PPARγ mRNA is expressed in all of the cell lines, albeit with a wide quantitative variation. LNCaP cells show the lowest amount of PPARγ mRNA whereas DU145 cells have an intermediate level. PC3 cells express PPARγ mRNA at levels that are even higher than those seen in the colon cancer cell line Moser, which was used as a positive control. We next examined the protein levels in all three cell lines. As shown in Fig. 1*C*, the differences observed at the gene expression level are also present at the level of the protein. Notably, despite high amounts of RNA in PC3 cells, a significant amount of the protein appears to be in the phosphorylated, inactive form that migrates more slowly (19).

PPARγ Mutation Analysis in Prostate Cancers and Cell Lines. To determine whether prostate cancers might have mutations in PPARγ, we analyzed LNCaP, DU145, and PC3 cell lines and 38 primary sporadic prostate adenocarcinomas of medium grade. The three cell lines did not harbor any mutations in this gene and

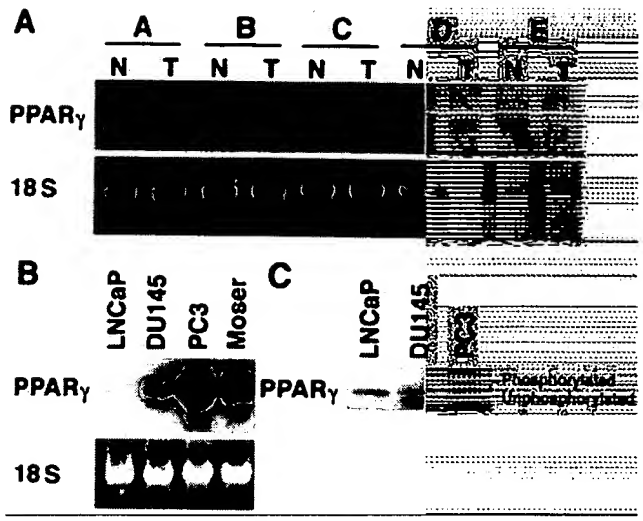

Fig. 1. PPARγ is expressed in human normal prostate, carcinomas, and cell lines. Expression of PPARγ mRNA in (*A*) samples from five patients (from A to E), obtained from normal prostate tissue (N), neighboring carcinomas (T), and (*B*) in LNCaP, DU145, and PC3 prostate cancer cell lines and colon cancer cells (Moser), used as control. (*C*) PPARγ protein levels in LNCaP, DU145, and PC3 cells.

no deletions in that chromosomal region were noted either. Further, none of the 38 primary prostate cancers were found to have intragenic PPARγ mutations. To determine whether this gene was deleted, loss of heterozygosity analysis was performed at D3S1263. As shown in Table 1, of the 38 tumors, 21 were informative at that locus and 40% were found to have hemizygous deletion of the PPARγ gene.

PPARγ Activation Inhibits the Growth of Prostate Cancer Cells. To investigate the role of PPARγ in human prostate cancer cells, we applied several ligands of different potency and selectivity to the cell line DU145 and determined the cell number after continuous treatment with these drugs. As shown in Fig. 2*A*, troglitazone caused a dose-dependent suppression of the growth of these cells, compared with vehicle-treated cells. The decrease occurred in a time- and dose-dependent manner, with a 50% inhibition detected at 7 days, at 10 μM concentration. LNCaP and PC3 cells also were treated with troglitazone at 10 μM. As shown in Fig. 2*B*, in both cell lines growth inhibition can be observed and the extent of this phenomenon appears to correlate with the levels of PPARγ protein present in these cells.

Next, DU145 cells were treated with 5 μM of other two PPARγ ligands, rosiglitazone and 15-deoxy-$\Delta^{12,\ 14}PGJ_2$, and cells were counted after 7 days of treatment. As shown in Fig. 2*C*, the maximum effects were seen with rosiglitazone, which showed a 70% inhibition of growth at 5 μM, compared with the vehicle-treated cells. The natural ligand 15-deoxy-$\Delta^{12,\ 14}PGJ_2$ gave greater than 60% growth inhibition under the same conditions. The profiles of growth responses to these agonists are consistent with their affinities to PPARγ and with their effects observed in adipocytes and other epithelial cells.

To critically determine whether cell growth inhibition is the result of PPARγ activation, we compared the growth response of DU145 to troglitazone with that elicited by M2, an inactive metabolite of troglitazone that is unable to bind and activate PPARγ. As shown in Fig. 2*C*, cells exposed to M2 did not show any decrease in number, compared with the vehicle-treated cells. We also treated these cells with ligands that activate specifically the other PPAR family members. Wy 14,464 is a specific activator of PPARα and GW233 is an activator of PPARδ, at 10 and 5 μM, respectively. The growth of the cells was not detectably affected by these ligands, suggesting that the inhibitory effects of PPARγ on cell growth do not extend to the other PPARs.

To determine whether the cells treated with these PPARγ ligands undergo programmed cell death or necrosis, we examined the propidium iodide (PI) staining of LNCaP, DU145, and PC3 cells by using cell sorting analysis. Despite the differences in cell number, 5 days of treatment with troglitazone or rosiglitazone at 10 μM did not cause a significant increase in PI positive staining (data not shown). These data suggest that changes in cell growth, but not cell death, account for the reduction in cell number observed after treatment with these drugs.

PPARγ Activation Decreases PSA Production of LNCaP Cells. PSA is a widely used marker for the diagnosis and management of patients with prostate cancer. In general, the level of serum PSA reflects tumor volume. To determine whether the activation of PPARγ can alter PSA levels, we measured PSA in supernatants from LNCaP cells after exposure to troglitazone. As shown in Fig. 3, the amount of PSA secreted per cell decreased as a result of treatment with troglitazone. After 48-h treatment with troglitazone, PSA secretion decreased by 4.7%, 24.9%, and 66.9% at 0.4, 2, and 10 μM troglitazone, respectively. PSA secretion continued to decrease with troglitazone treatment at 10 μM over the next 48 h by 75% compared with the values of the control cells. These data suggest that activation of PPARγ can exert a modulatory effect on the PSA expression of these cells.

Effects of PPARγ Activation on PSA Levels in Prostate Cancer Patients. To evaluate the effects of PPARγ activation in patients with androgen-dependent and androgen-independent prostate cancer, we conducted a phase II clinical study using the PPARγ ligand troglitazone. The baseline patient characteristics are summarized in Table 2. Forty-one men with histologically confirmed prostate cancer, and no symptomatic metastatic disease, were treated with troglitazone at 800 mg daily. The results of the treatment are summarized in Table 3. One patient with androgen-dependent prostate cancer had a decrease in PSA greater than 50% confirmed on multiple determinations. Interestingly, this PSA response was achieved after 16 months of treatment (Fig. 4*A*). No PSA decreases >50% were observed in patients with androgen-independent prostate cancer. PSA decreases less than 50%, confirmed on two determinations at least 4 weeks apart, were observed in 3/12 (25%) patients with androgen-dependent prostate cancer and 4/29 (14%) patients with androgen-independent prostate cancer.

Treatment with troglitazone was associated with long periods of stable disease characterized by the absence of new symptoms and no new metastases. The median duration of treatment was 18 weeks (range 5.4 to 90 weeks). For men with androgen-dependent prostate cancer, the median duration of treatment was 26.8 weeks (range 13 to 90 weeks). Among men with androgen-independent prostate cancer, the median duration of treatment was 14.3 weeks (range 5.4 to 54 weeks). The PSA

Table 1. Mutation and deletion analysis of PPARγ in prostate cancer cell lines and prostate primary carcinomas

	Mutations	Deletions
Cell lines (LNCaP, DU145, PC3)	None	None
Tumors		
Informative	0/21	8/21
Noninformative	0/17	0/17

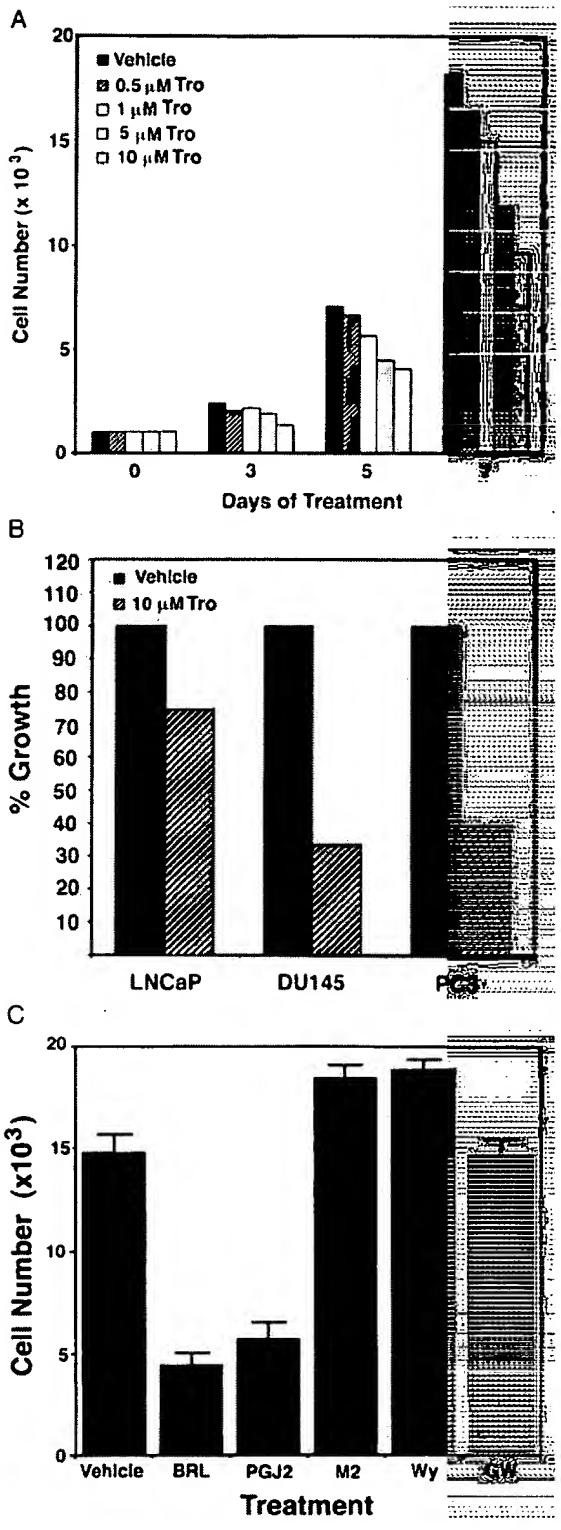

Fig. 2. PPARγ activation inhibits cell growth in prostate cancer cell lines. (*A*) Time course and dose-response inhibition of cell growth of DU145 treated with troglitazone. (*B*) Comparison of the growth inhibitory effects of troglitazone at 10 μM in LNCaP, DU145, and PC3 cells. (*C*) Growth inhibition in DU145 cells treated for 7 days with 5 μM rosiglitazone (BRL) and 5 μM 15-deoxy-$\Delta^{12,14}$PGJ$_2$ (PGJ2). DU145 treated with 10 μM M2, an inactive metabolite of troglitazone, 10 μM of Wy, or 5 μM of GW 00233, PPARα and PPARδ ligands, respectively, did not show any growth inhibition compared with troglitazone-treated cells.

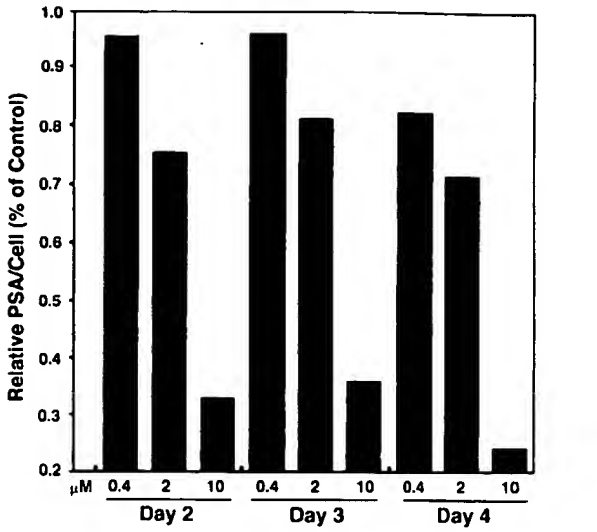

Fig. 3. PSA levels are decreased by troglitazone *in vitro*. LNCaP cells show decreased levels of PSA after treatment with different doses of troglitazone compared with vehicle-treated cells.

histories for the two patients with the longest PSA stabilizations are shown in Fig. 4. There were insufficient pretreatment data to evaluate the effect of troglitazone treatment on PSA velocity or PSA doubling time. Troglitazone treatment was tolerated well overall, except for one patient in whom a transient grade 3 elevation in transaminases was noted and another patient in whom reversible grade 3 diarrhea was observed. There were no other grade 2 or greater toxicities.

It has been shown that troglitazone treatment decreases total and free testosterone levels in women with polycystic ovary syndrome (23). To evaluate the effect of troglitazone treatment on gonadal steroids in men with androgen-dependent prostate cancer, we determined the levels of sex hormone binding globulin (SHBG), total and free testosterone, estradiol, lutenizing hormone (LH), and follicle stimulating hormone (FSH) at baseline and after 4 weeks of treatment. Treatment with troglitazone resulted in significant increases in SHBG and total testosterone levels with no significant change in the levels of free testosterone, estradiol, LH, or FSH (data not shown). These data suggest that changes in serum PSA seen after troglitazone treatment in men with androgen-dependent prostate cancer are mediated directly

Table 2. Baseline characteristics of the patients enrolled in the phase II clinical study

Age	
Median	70 years
Range	49–86 years
PSA	
Median	13.7 ng/ml
Range	1.0–919.6 ng/ml
Androgen dependent	12/41 (29%)
Androgen independent	29/41 (71%)
Metastases	16/41 (39%)
Bidimensionally measurable disease	0/41 (0%)
Gleason sum	
4–6	4/41 (11%)
7	14/41 (34%)
8–10	23/41 (55%)

Table 3. Changes in PSA levels in prostate cancer patients treated with troglitazone

Changes in PSA	Overall, $n = 41$	Androgen-dependent, $n = 12$	Androgen-independent, $n = 29$
>50% Decrease	1/41	1/12	0/29
<50% Decrease	7/41	3/12	4/29
No decrease	33/41	8/12	25/29
Median time on study	18+ weeks	20+ weeks	18+ weeks

through PPARγ rather than by an indirect effect of troglitazone on gonadal steroid levels.

Discussion

Inhibiting proliferation of cancer cells via induction of differentiation is an attractive approach to human cancer therapy. Although all-trans retinoic acid has been shown to induce differentiation and lead to clinical benefit in patients with acute promyelocytic leukemia (24), no treatment based on cell differentiation exists for human solid tumors. Given the role of PPARγ in inducing cell growth inhibition through a differentiation-like response in several cell lines and in a small clinical trial for liposarcoma (16), we attempted to extend this approach to human prostate cancer. The relatively low toxicity of PPARγ ligands adds considerable interest to this approach.

We show here that PPARγ is expressed in human prostate epithelium, both in normal tissue and carcinomas. PPARγ also is expressed in several of the common prostate cancer cell lines, albeit at varying levels. More importantly, activation of PPARγ by different classes of ligands influences the growth of prostate cancer cells. In particular, the ligand activation of this receptor inhibits cell growth in a dose-dependent manner. Importantly, we demonstrate that these effects are the specific consequence of PPARγ activation, because PPARα or PPARδ ligands do not show similar effects. To ensure that no intragenic PPARγ mutations occur in prostate cancers similar to those seen in colon cancers (17), which might interfere with ligand-related differentiation therapy, we examined the gene in 38 primary prostate cancers and the cell lines LNCaP, DU145, and PC3. None of these primary carcinomas or cell lines were found to have any intragenic mutation.

Encouraged by the results obtained *in vitro* and by the low toxicity of troglitazone, we decided to examine the effects of this drug in patients. Forty-one patients with metastatic prostate cancer were treated with 800 mg/day of troglitazone. Patients were monitored every 4 weeks by measuring serum PSA levels. Eight of 41 men experienced sustained PSA decreases after treatment. Twenty percent of patients had sustained serum PSA decline between 1% and 50%. Perhaps more impressively, a significant fraction (39%) of patients demonstrated prolonged stabilization of their serum PSA. In one patient, troglitazone treatment resulted in a dramatic fall in serum PSA (98%) below baseline levels. The drug was extremely well tolerated with no side effects related to the treatment except for transient liver function test abnormalities seen in one patient and diarrhea in another.

The interpretation of these data are complex for several reasons. The effects on serum PSA observed in a fraction of patients could reflect primary changes in the relative expression of the PSA gene per cell or changes in the rate of cell growth. The changes that occur immediately after this drug is given may reflect an alteration in PSA synthesis and secretion per unit of tumor. However, changes in gene transcription rates generally occur over hours or days at the most. The very prolonged stabilization of PSA values in a subset of patients is more likely

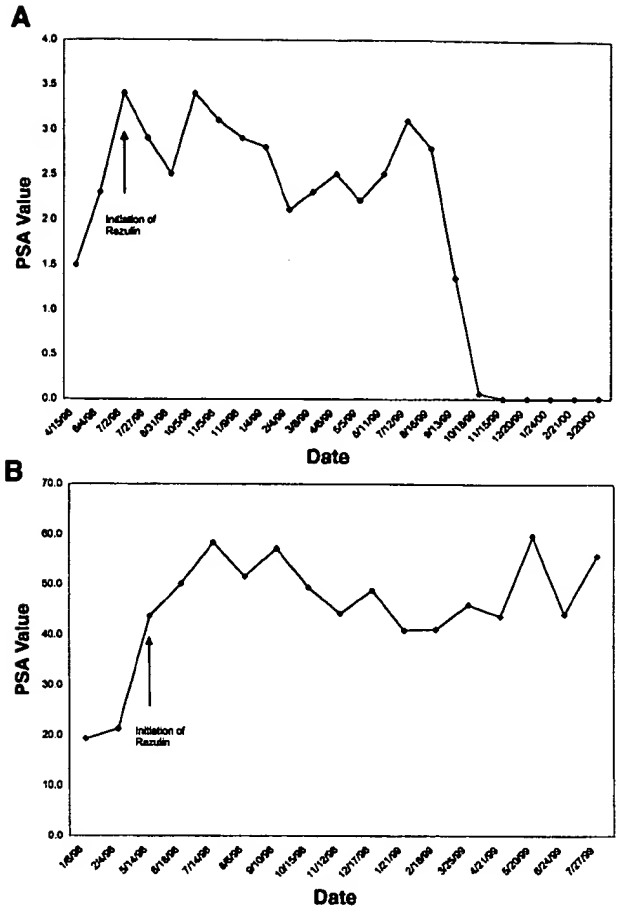

Fig. 4. PSA levels are modulated by troglitazone in prostate cancer patients. The PSA levels of prostate cancer patients, treated with 800 mg of troglitazone/day, were measured every 4 weeks. (A) The patient received radiation therapy in 11/94, nadir of 0.1. (*B*) Patient had post radiation therapy nadir of 1.9 on 6/2/94.

to reflect a prolonged change in the growth status of the tumor in these patients. This possibility is supported by the clinical status of these patients. None of the patients became symptomatic during the treatment period. Data on a control group, obtainable in a larger clinical trial, are needed to confirm this trend.

These results suggest that targeting PPARγ for the treatment of prostate cancer may be useful for at least a certain subset of patients. Markers for biologic effects are currently lacking. How the patients who had stable PSA values in response to troglitazone differ from those who didn't remains unclear. However, our previous studies with breast cancer cells (10) suggested that tumors having high levels of active mitogen-activated protein (MAP) kinase may exhibit *de novo* resistance to PPARγ ligands. MAP kinase is known to modify and suppress PPARγ function (19).

Because mitogen-activated protein kinase, a downstream target of activated ras, often is elevated also in prostate cancer (25), it is possible that it could contribute to resistance also in this context. Another biologic marker for response is the status of the gene encoding PPARγ itself. For example, in colon cancers, all mutations found compromised the ability of this receptor to respond to ligands (17). Although none of the 38 prostate cancer samples analyzed had intragenic mutations,

almost half of the informative tumors were found to carry hemizygous deletions in this region. It is possible that hemizygosity for PPARγ predicts for a poorer or less durable response. This finding also would confirm that haploinsufficiency can contribute to the reduction in the tumor suppressing effect of this receptor, as originally postulated for colon cancers (17). Both somatic and germ-line profiling of PPARγ in the context of response to ligands must be pursued in the future as an important pharmacogenomic issue.

It is possible that treatment with any effective activator of PPARγ could provide an additional useful agent in the treatment of prostate cancer. This approach is especially attractive because of the mild side effect profile associated with PPARγ ligands. Larger phase II and phase III studies with clinical endpoints are needed to confirm the biologic activity of PPARγ ligands and to delineate subsets of patients who may benefit from such treatments.

We thank Wendy Smith for technical assistance. This work was supported by a CapCure grant to B.M.S. and P.W.K. and by National Cancer Institute Grant P30CA16058 and the Mary Kay Ash Charitable Foundation to C.E.

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SW 982 Cells Troglit ± Cisplatin ± 5-Fluorouracil 030

	Troglit	FU	cisP
3	5 µg/ml	–	–
6	10 µg/ml	–	–
9	–	1 µM	
12	+	10	
15	+	~~500~~ 100	
18	+ 10 µg/ml	1	
21	+ Troglit	10	
24	+	~~500~~ 100	
27	+	–	1
30	+	–	10
33	+	–	~~80~~ 50
36	+	–	1
39	+	–	10
42	+	–	500 6 µl Dmso/ml
45	6 µl Dmso/ml		
48	—	–	–

Stock preparation
5-Fluoro uracil 50 mM DMSO IC50 4.5 µM
Cis-platinum 10 mM DMSO

Tests plate - 4 - 12 well plates
Thur - Rx
Mon - Measure DNA LDH IC50 per media

STD	Reads	ng/mL
50	49	
100	198	
200	257	
300	325	

Q. 30 min

DNA Trig SFU cisPL

4 x 4 x 10

TV=1ml	1	397	No Rx	25	52	1 μM cisPlat	
1-48	2	265	①	26	54		
	3	360		27	51		
	4	110	10 μg Troglit	28	36	10 μM cPl	
	5	146	②	29	33		
	6	131		30	44		
	7	143	1 μM 5FU	31	14	50 μM cPl	
	8	99	③	32	15		
	9	82		33	13		
	10	95	10 μM FU	34	63	10 μg/ml Trog	
	11	111	④	35	66	1 μM cPl	
	12	61		36	56		
	13	41	100 μM FU	37	36	Trog / 10 μM cPl	
	14	37	⑤	38	57		
	15	49		39	42		
	16	41	10 μg/ml Troglit	40	15	Troglit / 50 μM cPL	
	17	44	⑥ 1 μM 5FU	41	13		
	18	36		42	11		
	19	30	Trog / 10 μM FU	43	133	No Rx 5% DMSO	
	20	48	⑦	44	167		
	21	48		45	122		
	22	24	Trog / 100 μM FU	46	156	No Rx	
	23	23	⑧	47	172		
	24	17		48	166		

TV=5ml Top 2/3 Sol 1

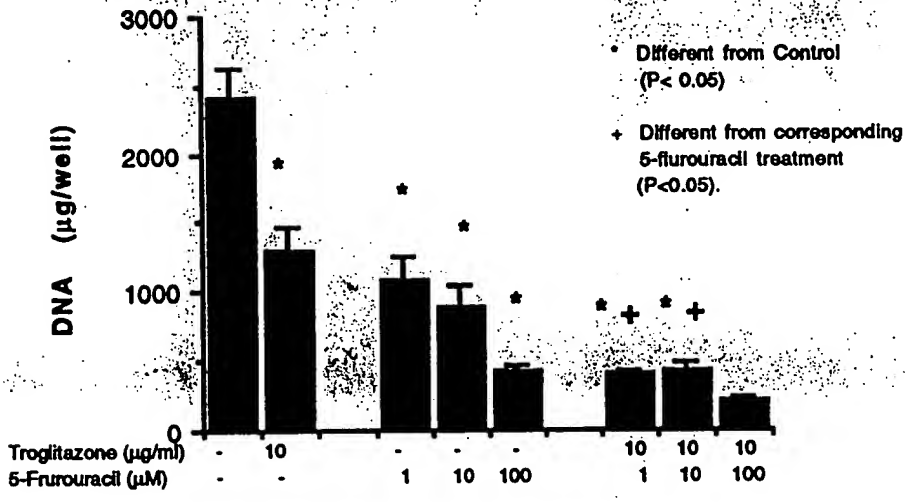
Hist	1	486
	2	458
	3	455
	4	32
	5	27
	6	23

TV=1ml

Hist	1	682
FBS Dose	2	752
	7	661
	8	406

P-30 WDN

DNA CONTENT OF SAOS-2 CELLS 3 DAYS AFTER A SINGLE TREATMENT OF TROGLITAZONE AND/OR 5-FLUROURACIL



LDH ACTIVITY IN SAOS-2 CELLS 3 DAYS AFTER A SINGLE TREATMENT OF TROGLITAZONE AND/OR 5-FLUROURACIL